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(54) Title: METHOD FOR DIAGNOSIS OF OVARIAN DYSGENESIS

(57) Abstract

A method is provided for determining a follicle stimulating hormone receptor genotype in a human patient, which method is particularly useful for diagnosing ovarian dysgenesis in affected human females. The method comprises analysis of DNA from a patient which encodes all or a portion of the receptor for follicle-stimulating hormone.

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METHOD FOR DIAGNOSIS OF OVARIAN DYSGENESIS

FIELD OF THE INVENTION

The present invention relates to methods for the detection of hereditary ovarian dysgenesis.

BACKGROUND OF THE INVENTION

Normal gonadal function depends upon the integrity of the pituitary-gonadal axis. In females, regulatory control of the ovary is primarily accomplished by the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). At birth, development of the ovarian follicles is normally arrested until pituitary gonadotropin stimulation at puberty causes follicular maturation. Either the failure of the pituitary to secrete sufficient amounts of FSH and/or LH or the failure of the ovary to respond to gonadotropin stimulation results in hypogonadism. In females, such a condition may be characterized by the development of anatomically normal internal and external genitalia, variably developed secondary sexual characteristics, and amenorrhea. Failure of the ovaries to respond appropriately to FSH and/or LH stimulation results in poorly-developed (streak) ovaries and increased circulating levels of the hormone(s) to which the ovary is not responding. Such a condition is referred to as ovarian dysgenesis. That disease is one of a group of diseases characterized by hypogonadism which are often referred to generally as hypergonadotropic hypogonadisms. Many such diseases are due to mutations in the sex chromosomes. However, ovarian dysgenesis in females with an XX karyotype is rare and may be due to an autosomal recessive mutation. Simpson, *et al., Birth Defects: Original Article Series, 7*:215-228 (1971); Aittomäki, *Am. J. Genet., 54*:844-851 (1994).

Also of interest to the present invention are the receptors for FSH and LH. Each of those receptors is a member of a family of receptors which are coupled to GTP-binding proteins (G-proteins) in order to effect intracellular signalling. Other members of this family include certain adrenergic receptors, muscarinic cholinergic receptors, vasopressin receptors, and angiotensin receptors. Most receptors in this family comprise three distinct domains, an extracellular domain, a

transmembrane domain typically having seven membrane-spanning regions with six intervening loops, and an intracellular carboxy-terminal domain. The transmembrane domain is highly conserved in G-protein coupled receptors. However, LH and FSH are distinguished from other members of the family by their relatively large extracellular ligand binding domain.

While ovarian dysgenesis has been studied at the hormonal level, no causative mutation has been proposed and no screening method for diagnosis of ovarian dysgenesis has been available. Accordingly, there is a need in the art for an accurate, reliable method for diagnosing ovarian dysgenesis as provided by the present invention.

SUMMARY OF THE INVENTION

The invention provides a method for diagnosing ovarian dysgenesis genotypes and conditions based on polynucleotide sequence differences between wild type alleles of the follicle-stimulating hormone receptor (*fshr*) gene and alleles of the gene that are associated with hypergonadotropic hypogonadisms such as ovarian dysgenesis. The method of the invention can identify individuals (male or female) that are genetic carriers as well as identify patients that exhibit (or, upon maturation, will exhibit) a particular hypergonadotropic hypogonadism. Any methodologies for analyzing polynucleotides are contemplated for analyzing polynucleotides according to the method of the invention, including sequencing techniques, techniques exploiting restriction mapping of nucleic acids to reveal restriction pattern differences, and nucleic acid hybridization strategies designed to detect polynucleotide sequence differences as small as a single nucleotide difference. Of course, any of these techniques may be combined with additional techniques to facilitate the practice of the methods of the invention, such as amplification of the nucleic acids in a biological sample by, *e.g.*, the polymerase chain reaction (PCR) technique.

In a preferred embodiment, a method for diagnosing ovarian dysgenesis in a patient (*e.g.*, a female patient) comprises the steps of obtaining a cell sample from a patient, isolating nucleic acids from the cell sample; amplifying a portion of the nucleic acids encoding a receptor for follicle-stimulating hormone,

thereby generating amplified DNA; exposing the amplified DNA to a restriction endonuclease selected from the group consisting of *Bsm*I and restriction endonucleases having a recognition site which overlaps that of *Bsm*I, under conditions wherein the restriction endonuclease specifically cleaves DNA at its recognition site; detecting polynucleotide restriction fragments of the amplified DNA; and diagnosing ovarian dysgenesis from the restriction fragments, wherein ovarian dysgenesis is correlated with a C to T mutation in codon 189 of the DNA encoding a receptor for follicle-stimulating hormone, the mutation eliminating a recognition site of the restriction endonuclease. The enzyme *Bsm*I recognizes the sequence 5'-NG'CATTC-3' and cleaves between the indicated G and C. Restriction endonucleases that recognize sequences which overlap the recognition site of *Bsm*I, such as *Sac*III or *Sst*III, may also be used. *Sac*III and *Sst*III recognize sequences internal to a *Bsm*I site.

The portion of nucleic acids subjected to amplification need not include the entire coding sequence for a follicle-stimulating hormone receptor. For example, the portion of nucleic acids amplified in the method of the invention may include only exon 7, or only a portion of exon 7. Depending upon the particular portion of nucleic acid amplified in the method of the invention, and the particular restriction enzyme used, the number and/or size of restriction fragments may vary. For example, using nucleic acids comprising exon 7 of a follicle-stimulating hormone receptor (*fshr*) allele and *Bsm*I, a diagnosis of ovarian dysgenesis is made if the amplified nucleic acid remained uncut when exposed to the restriction endonuclease (*i.e.*, a single fragment comprising the uncut amplified nucleic acid is observed).

In another preferred embodiment, the invention provides a method for diagnosing ovarian dysgenesis in a female patient suspected of having ovarian dysgenesis, comprising the steps of obtaining a cell sample from the female patient; isolating nucleic acid from the cell sample; amplifying a portion of the isolated nucleic acid comprising exon 7 of a follicle-stimulating hormone receptor gene, thereby generating amplified DNA; sequencing the amplified DNA; comparing the sequence of the amplified DNA to SEQ ID NO:1; and diagnosing ovarian dysgenesis by the presence of one or more sequence differences between the sequence

of the amplified DNA and SEQ ID NO:1. The sequence differences, for example, may involve a single nucleotide difference at position 640 of SEQ ID NO:1 (*e.g.*, a thymidylate in the amplified DNA corresponding to the cytidylate at position 640 of SEQ ID NO:1).

Another aspect of the invention is directed to a method for determining a follicle-stimulating hormone receptor (*fshr*) genotype in a human patient, which comprises the steps of providing a biological sample comprising nucleic acid from the patient, the nucleic acid including the patient's *fshr* alleles; analyzing the nucleic acid for the presence of a mutation or mutations in codon 189 of the *fshr* alleles; and determining an *fshr* genotype from the analyzing step, wherein the presence of a mutation in codon 189 of a *fshr* allele is correlated with an ovarian dysgenesis genotype. The method is useful for screening both male and female patients for determining an *fshr* genotype, for predicting/diagnosing disease states (e.g., ovarian dysgenesis in a female patient), and for genetic counseling purposes (e.g, for couples intending to have children). The biological sample may be any sample from the patient containing nucleic acid suitable for analysis (e.g., tissue and fluid samples); a cell sample is a preferred biological sample. Further, the analyzing step of the invention embraces a variety of analytical techniques including sequencing all, or a portion, of a nucleic acid (which may be DNA or RNA) comprising codon 189 of a *fshr* allele. By way of further illustration, the analyzing step of the invention includes restriction endonuclease analyses, wherein a nucleic acid is exposed to a restriction endonuclease having a recognition site that includes codon 189 of a wild type *fshr* allele, under conditions wherein the restriction endonuclease specifically cleaves DNA at its recognition site; and detecting the nucleic acid or polynucleotide restriction fragments thereof resulting from the exposing step. Restriction endonucleases for use in this embodiment of the invention include *Bsm*I, restriction endonucleases recognizing sites that overlap a *Bsm*I site, restriction endonucleases recognizing the same cleavage sites as *Bsm*I, and isoschizomers of *Bsm*I.

In another aspect of the invention, a method for screening for an ovarian dysgenesis genotype in a patient comprises the steps of providing a biolo-

gical sample comprising nucleic acid from the patient, the nucleic acid including the patient's follicle-stimulating hormone receptor (*fshr*) alleles; amplifying a portion of the nucleic acid to generate amplified DNA, the portion including codon 189 of the *fshr* alleles; exposing the amplified DNA to a restriction endonuclease selected from the group consisting of *Bsm*I restriction endonuclease and restriction endonucleases having a recognition site which overlaps a *Bsm*I recognition site, under conditions wherein the restriction endonuclease specifically cleaves DNA at the recognition site of the restriction endonuclease; thereafter detecting said amplified DNA or restriction fragments thereof; comparing the amplified DNA or fragments thereof to control nucleic acid of a human subject free of an ovarian dysgenesis genotype, wherein said control nucleic acid has been amplified, exposed, and detected as described above; and screening for an ovarian dysgenesis from the comparison, wherein an ovarian dysgenesis genotype in the patient is correlated with a different number of detected amplified DNA or restriction fragments thereof from the patient's amplified DNA than from the control amplified DNA. In screening for ovarian dysgenesis genotypes, for example, a homozygous ovarian dysgenesis genotype may be correlated with fewer detected restriction fragments from the patient's amplified DNA than from the control amplified DNA. Consistently, the screening may correlate a heterozygous ovarian dysgenesis genotype with a greater number of detected amplified DNA or restriction fragments thereof from the patient's amplified DNA than from the control DNA.

Yet another aspect of the invention is a method comprising the steps of obtaining a cell sample from a female patient; isolating DNA from the cell sample; amplifying a portion of the DNA encoding exon 7 of the follicle-stimulating hormone receptor; exposing the amplified DNA to *Bsm*I or another restriction endonuclease which recognizes the *Bsm*I site; and diagnosing ovarian dysgenesis as the presence of a single fragment upon digestion.

Additional aspects of the invention will become apparent upon consideration of the following detailed description thereof.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the human FSH receptor showing the multi-exon structure of the gene encoding the protein; the extracellular (Ec), transmembrane (Tm), and intracellular (Ic) domains of the receptor; and the Ala to Val and Asn to Ser transitions (boxed) described herein.

Figure 2 shows a pedigree of families used in studies of ovarian dysgenesis inheritance patterns.

Figure 3 is a map of microsatellite markers on human chromosome 2.

Figure 4 depicts the nucleotide sequence, and deduced amino acid sequence, of the FSH receptor (SEQ ID NO:1) gene, showing *Bsm*I sites in bold.

Figure 5 is a graph showing results of FSH-induced cAMP production by MSC01 cells transfected with wild-type or mutant FSH receptor-encoding DNA.

Figure 6 is a graph showing Scatchard analysis of FSH binding to MSC-1 cells transfected with wild-type (circles) or mutated (triangles) FSH receptor-encoding DNA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for the diagnosis of diseases characterized by hypergonadotropic hypogonadisms such as ovarian dysfunction. In particular, methods of the invention are useful in the diagnosis of ovarian dysgenesis in females. Diagnostic methods according to the invention may be used at any time in the life of the patient, even prior to the onset of symptoms, such as the failure of normal onset of menstruation at puberty. Methods of the invention are primarily based upon the discovery that a mutation in the coding sequence of the gene for the follicle-stimulating hormone receptor accounts for the inability of FSH to generate signal transduction at its receptor.

In the normal genotype for the follicle-stimulating hormone receptor, four *Bsm*I restriction sites (5'-NGCATTC-3') exist, as shown in Figure 4 (SEQ ID NO:1). In individuals having ovarian dysgenesis, the FSH receptor gene has been observed to have a mutation at position 640 (SEQ ID NO:1), such that a thymine

is substituted for a cytosine, resulting in the sequence, 5'-NGTATTC-3'. This substitution eliminates the *Bsm*I site spanning nucleotides 638-644 in the normal (wild type) fshr gene. Thus, digestion of DNA encoding a wild-type FSH receptor with *Bsm*I normally produces five fragments (due to cleavage at all four *Bsm*I sites in a linear DNA). However, the thymine-to-cytosine mutation at position 640 results in a mutated DNA that produces only four restriction fragments upon *Bsm*I digestion. As shown below, mutation at position 640 is uniquely associated with ovarian dysgenesis; whereas no mutation at any of the other three *Bsm*I sites in the FSH receptor DNA has been found in patients suffering from that disease. Accordingly, *Bsm*I digestion and subsequent observation of the products produced is useful as a diagnostic method or a screening method for ovarian dysgenesis. As detailed below, position 640 in SEQ ID NO:1 falls within exon 7 of the fshr gene. In one embodiment of the invention, the region of exon 7, or a portion thereof, of the FSH receptor coding sequence may also be isolated and exposed to *Bsm*I. Exon 7 contains a unique *Bsm*I site which, if mutated, will produce no *Bsm*I digestion products, thereby enabling diagnosis of ovarian dysgenesis.

The gene encoding the FSH receptor has been mapped to human chromosome 2p16-21. Rousseau-Merck, *et al., Genomics, 15*:222-224 (1993). The FSH receptor coding sequence is shown in SEQ ID NO:1 and in Figure 4 and is available in the Genbank database as Accession No. S59900. Figure 1 shows a schematic of the FSH receptor showing the regions encoded by the 10 exons.

The following examples illustrate preferred means for detecting FSH receptor genotypes generally and for diagnosis of ovarian dysgenesis in human females.

EXAMPLE 1

Venous blood samples were obtained from 37 individuals, each belonging to one of 6 families having at least two females with symptoms of ovarian dysgenesis. Total DNA was isolated from fresh white blood cells or lymphoblastoid cell lines established from the samples. The method of Chomczynski, *et al., Anal. Biochem., 162*:156-159 (1987), incorporated by reference herein, was

used to isolate RNA from the samples. A pedigree showing inheritance patterns in individuals used to isolate the ovarian dysgenesis locus is shown in Figure 2.

Linkage was investigated using Généthon microsatellite markers as reported in Weissenbach, *et al., Nature, 359*:794-801 (1992), incorporated by reference herein. A linkage map for chromosome 2 is provided in Figure 3. Forty-seven markers were chosen at approximately 20 centimorgan (cM) intervals. Pairwise linkage analyses were carried out using the MLINK subprogram of the LINKAGE program package reported in Lathrop, *et al., Proc. Nat'l. Acad. Sci. (USA), 81*:3443-3446 (1984), incorporated herein by reference. The disease allele frequency was set to 0.01 and lod scores were computed under a model of equilibrium between the disease locus and each marker locus, assuming a recessive mode of inheritance with full penetrance in females. A lod score is defined as the $\log_{10}$ of the ratio of the probability that the data obtained would have arisen from unlinked loci and represents a criterion for assigning a given restriction fragment length polymorphism to a particular linkage group. Linkage was confirmed using the G2113A transition located in exon 10 of the FSH receptor gene as a polymorphic marker (see below). Amplification was accomplished using PCR according to the method of Sankila, *et al., Hum. Mol. Genet., 4*:93-98 (1995), incorporated by reference herein.

Preliminary evidence of linkage of the ovarian dysgenesis phenotype was noted for markers D2S134 and D2S177 on chromosome 2. Linkage was confirmed using markers D2S119 and D2S123, each of which is located between D2S134 and D2S177. Markers D2S119 and D2S123 define an approximately 12 cM region of chromosome 2. That region was further studied using markers D2S391, D2S288, CA21 and CA7, each of which is located in the 12 cM region defined above, and linkage of the ovarian dysgenesis locus to this region of chromosome 2 was confirmed.

Pairwise linkage analyses produced 46 potentially informative meioses, of which 30 were from affected females. The lod scores obtained from those meioses revealed no recombinations between the markers and the ovarian dysgenesis locus as evidenced by phenotype. The highest lod score, $Z_{max} = 4.71$, was obtained for marker D2S391, while two other marker loci, D2S288 and CA21,

provided lod scores exceeding the limit of proven linkage ($Z \geq 3.0$). The region in which linkage of the ovarian dysgenesis phenotype exists was, therefore, isolated to the 2p region of chromosome 2 based upon the above linkage analysis. That region of chromosome 2 is the region which contains the genes encoding the receptors for follicle-stimulating hormone and luteinizing hormone. Thus, it was hypothesized that a nonlethal mutation in one of these two genes was responsible for ovarian dysgenesis. On the basis that an LH receptor mutation would also cause severe hypogonadism in males and no males were affected in the pedigrees producing affected females, the LH receptor gene was considered unlikely as a site of mutation causing ovarian dysgenesis. Accordingly, the gene encoding the FSH receptor was investigated as a source of the ovarian dysgenesis phenotype.

EXAMPLE 2

As shown in Figure 1, the FSH receptor gene comprises 10 exons. As recited above, the polynucleotide sequence of the entire *fshr* coding region can be obtained from Genbank under Accession Number S59900. The polynucleotide sequence of each one of the ten exons of *fshr* has also been deposited under individual accession numbers in the Genbank database. A comparison of the sequences of the individual exons to the coding region of the entire coding region presented in SEQ ID NO:1 reveals that a few terminal nucleotides from each exon are lost in the process of forming a mature mRNA during expression. The correlation between the sequences of the individual exons and the sequence presented in SEQ ID NO:1 follows. Nucleotides 1-158 of exon 1 (Acc. No. X91738) are found at positions 70-227 of SEQ ID NO:1; nucleotides 7-77 of exon 2 (Acc. No. X91739) span positions 228-298 of SEQ ID NO:1; nucleotides 6-80 of exon 3 (Acc. No. X91740) are found at positions 299-373 of SEQ ID NO:1; nucleotides 6-82 of exon 4 (Acc. No. X91741) span positions 374-450 of SEQ ID NO:1; nucleotides 8-77 of exon 5 (Acc. No. X91742) are found at positions 451-520 of SEQ ID NO:1; nucleotides 6-83 of exon 6 (Acc. No. X91743) span positions 521-598 of SEQ ID NO:1; nucleotides 6-75 of exon 7 (Acc. No. X91744) are found at positions 599-668 of SEQ ID NO:1; nucleotides 7-80 of exon 8 (Acc. No. X91745) span positions 669-742 of SEQ ID NO:1; nucleotides 6-191 of exon 9 (Acc. No.

X91746) are found at positions 743-928 of SEQ ID NO:1; and nucleotides 102-1352 of exon 10 (Acc. No. S73526) span positions 929-2179 of SEQ ID NO:1.

A systematic analysis was performed to determine the change or changes in that gene which are responsible for expression of the ovarian dysgenesis phenotype.

The large terminal exon of the FSH receptor was screened for mutations using denaturing gradient gel electrophoresis. Exon 10 was amplified with GC clamped primers in four different PCR reactions with overlapping products covering the entire transmembrane and intracellular domains. Pairs of GC clamped primers used in PCR were primer 10f: 5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCGGACTTATGCAATGAAGTGGTTG-3' (forward, SEQ ID NO:2), 10r: 5'-GTGAAAAAGCCAGCAGCATC-3' (reverse, SEQ ID NO:3); primer 11f: 5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCGATTGACTGGCAAACTGGGG-3' (forward, SEQ ID NO:4), 11r: 5'-AGAGGAGGACACGATGTTGG-3' (reverse, SEQ ID NO:5); primer 12f: 5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCGGGCTGCTATATCCACATCTACC-3' (forward, SEQ ID NO:6), 12r: 5'-CAGAACCAGCAGAATCTTTGC-3' (reverse, SEQ ID NO:7); and primer 13f:5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCGCTTTCTTTGCCATTTCTGCC-3' (forward, SEQ ID NO:8), 13r 5'-CAAAGGCAAGGACTGAATTATC-3' (reverse, SEQ ID NO:9). Each pair of primers was optimized for each fragment with the MELT 87 program described in Lerman, *et al., Methods in Enzymology* (Wu, *et al.*, eds. 1989), incorporated by reference herein. For each PCR run, samples were heated to 94°C for 4 minutes and put through 34 cycles. For each cycle, denaturation was conducted for 1 minute at 94°C, annealing was conducted for 1 minute at 57°C, 58°C, 57°C, and 54°C for primer pairs 10, 11, 12, and 13, respectively, and extension was for 30 seconds at 72°C.

A 7% polyacrylamide gel was designed for each fragment with a linear concentration gradient of formamide. Gels were run in an aquarium at a stable temperature of about 60°C for 16-20 hours in a denaturing gel electrophoresis system, model DGGE2000 (C.B.S. Scientific Co.). Denaturing gradient gel electrophoresis procedures are generally known in the art and are available, for

example, in Ausubel, *et al.* (eds.) *Current Protocols in Molecular Biology, 2.12, et seq.* (1987), incorporated by reference herein.

A sequence polymorphism was detected in the terminal region of exon 10. A 326 bp fragment beginning at nucleotide 1892 of the FSH receptor coding sequence was amplified using primers 14f: 5'-AGCAAAGATTCTGCTGGTTC-3' (forward, SEQ ID NO:10) and 14r: 5'-CAAAGGCAAGGACTGAATTATC-3' (reverse, SEQ ID NO:9). The amplified PCR product was sequenced using the dideoxy chain termination method as reported in *Molecular Cloning: A Laboratory Manual* (Sambrook, *et al.*, eds 1989), incorporated by reference herein. A G-to-A transition was observed in the sequenced product at nucleotide position 2113 (G2113A transition). Position 2113 of SEQ ID NO:1 is in the region encoding the intracellular domain of the FSH receptor and predicts a change from Ser to Asn at amino acid position 680 of the corresponding protein structure. The G2113A transition abolishes a *Bsr*I site, thus enabling *Bsr*I to be used as a screen for the two alleles. The polymorphism at that site was designated FSHR1.

The G2113A transition observed in exon 10 of the FSH receptor coding sequence had previously been identified as a difference between the ovarian and testicular forms of the gene encoding the FSH receptor. Kelton, *et al., Mol. Cell. Endocrinol., 89*:141-151 (1992). Denaturing gradient gel electrophoresis analysis of samples obtained from a family having females both with and without ovarian dysgenesis showed that the allelic form of the gene at the G2113A transition had no affect on phenotype. Thus, the allelic variance at position 2113 was not considered to be disease causing. No recombinations between that locus and the disease phenotype were observed, as shown in Table 1.

TABLE 1

	Recombination fraction, Θ							
Marker locus	0.00	0.001	0.01	0.05	0.10	0.20	0.30	90% support interval
D2S119	2.41	2.41	2.35	2.12	1.82	1.20	0.62	0-0.17
D2S391	4.71	4.70	4.59	4.14	3.56	2.38	1.26	0-0.09
D2S288	3.10	3.10	3.03	2.72	2.33	1.52	0.78	0-0.13
CA21	4.20	4.19	4.11	3.74	3.24	2.19	1.16	0-0.10
CA7	2.66	2.66	2.60	2.35	2.03	1.36	0.71	0-0.16
D2S123	2.68	2.67	2.62	2.39	2.08	1.44	0.79	0-0.16
FSHR1	2.72	2.71	2.66	2.44	2.14	1.49	0.82	0-0.16

The FSHR1 locus was, however, used as a marker in the linkage analysis described above. Since the polymorphism in exon 10 was excluded as the source of the ovarian dysgenesis phenotype, the other exons of the FSH receptor were screened.

EXAMPLE 3

Exons 1-5 and 9 were screened by amplifying each with flanking intronic primers and sequencing the PCR product. None of those exons showed a mutation associated with the ovarian dysgenesis phenotype.

Complete sequences for the flanking introns of exons 6, 7, and 8 were not available. However, it was determined that FSH receptor-encoding mRNA could be isolated by using RNA from blood leukocytes as a template. Such a process takes advantage of so-called illegitimate transcription, whereby small amounts of mRNA encoding most tissue-specific proteins are produced by white blood cells. Due to the sensitivity of PCR and since PCR products can be directly sequenced, small amounts of RNA produced by white blood cells were used to amplify the FSH receptor coding region. RNA was isolated from white

blood cells and exons 6-9 were amplified by reverse transcription PCR in order to identify mutations by direct sequencing of the PCR products as follows.

Total RNA was isolated from white blood cells or from lymphoblasts. Approximately 0.8 μg RNA was used as a template for first-strand cDNA synthesis and primed for reverse transcription by 40 pmol of primer 15r: 5'-TAGTTTTGGGCTAAATGACTTAGAGGG-3' (SEQ ID NO:11), which is complementary to nucleotides 2161-2135 of SEQ ID NO:1. Approximately 1mM each of dATP, dCTP, dTTP, dGTP and 200 U M-mLv reverse transcriptase were combined in M-mLv reverse transcriptase buffer (Promega, Madison, WI) to a final volume 20 μL. Samples were incubated at 42°C for 1 hour. Samples were then heated to 95°C for 10 minutes and a 5 μl aliquot of the resulting cDNA product was used as a template for PCR.

The cDNA was amplified in two rounds of PCR using a nesting strategy in order to increase yield and specificity. In the first round of PCR, primer 15r (see above) and primer 16f: 5'-CCTGCTCCTGGTCTCTTTGCTG-3' (SEQ ID NO:12) were used. The reaction was heated for 2 minutes at 94°C and put through 20 cycles each comprising 1 minute denaturing at 94°C, 1 minute annealing at 58°C and 2 minutes extension at 73°C to produce a 2082 bp product. The product contained exons 6-9 and 5μl was used as a template for amplification of exon 7 using primer 6f: 5'-AGAAATTCTTTCGTGGGGCT-3' (forward, SEQ ID NO:13) and 6r: 5'-GTTTGCAAAGGCACAGCAAT-3' (reverse, SEQ ID NO:14). The resulting PCR product was a 357 bp fragment corresponding to nucleotides 558-914 of SEQ ID NO:1.

The 357 bp product, which included exon 7, was sequenced. Upon inspection of the resulting sequence in a number of patients (both affected and unaffected), it was noticed that all affected individuals were homozygous for a C-to-T transition at nucleotide 640 (C640T transition) of the FSH receptor sequence shown in SEQ ID NO:1. That change predicts an Ala-to-Val substitution at amino acid position 189 in the corresponding protein primary structure. The C640T transition segregated perfectly with the disease phenotype and all affected individuals were determined to be homozygous recessive for the mutation from cysteine to thymine. The mutation at position 640 abolishes a *Bsm*I restriction site, thus

enabling diagnosis of the disease by digestion of FSH receptor DNA with *Bsm*I. Thus, it was determined that the C640T transition in exon 7 of the FSH receptor gene is predictive of ovarian dysgenesis in affected individuals.

EXAMPLE 4

Digestion of genomic DNA with *Bsm*I was next used in order to confirm the use of that enzyme in the diagnosis of ovarian dysgenesis.

Genomic DNA isolated from 15 affected and 22 unaffected individuals was amplified by PCR by heating for 12 minutes at 94°C followed by 30 cycles at 94°C for 1 minute, 54°C for 1 minute, and 72°C for 2 minutes using primers 7f: 5'-GTTATTTCAGATGGCTGAATAAG-3' (SEQ ID NO:15) and 7r: 5'-GCTCATCTAGTTGGGTTC-3' (SEQ ID NO:16). These primers were selected to amplify a 78 base pair portion of genomic DNA encompassing a portion of the intron between exons 6 and 7 and most of exon 7 of the follicle stimulating hormone receptor gene. Exon 7 of the wild type *fshr* gene contains a unique *Bsm*I site at nucleotides 638-644 of SEQ ID NO: 1 which is abolished by the C640T transition observed in individuals with the ovarian dysgenesis phenotype (the three additional *Bsm*I sites in the FSH receptor coding sequence shown in Fig. 4 are not in exon 7 and are not included in the above-described PCR product). Fifteen microliters of the resulting 78 bp PCR product was digested to completion with 20 IU of *Bsm*I (Promega). The product of digestion was fractionated on a 10% non-denaturing polyacrylamide gel and the DNA was visualized with ethidium bromide.

In unaffected individuals, the PCR product is cleaved into 51 bp and 27 bp fragments by *Bsm*I. However, in affected individuals, only a 78 bp fragment appears in the gel. Heterozygous individuals, such as the parents shown in the Family 7 pedigree in Figure 2, show all three fragments. Thus, *Bsm*I digestion is probative of ovarian dysgenesis by detecting the C640T transition. The pathogenic role of the C640T transition was further confirmed in FSH receptor signalling and binding studies.

EXAMPLE 5

Human FSH receptor-encoding DNA was obtained by reverse transcription PCR using testicular poly (A^+) RNA as reported in Gromoll, *et al.*, *Biochem. Biophys. Res. Commun.*, *188*:1077-1083 (1992), incorporated by reference herein. The resulting PCR product was subcloned into a pBluescript SR(-) vector (Stratagene Inc., La Jolla, CA). A 5′ untranslated region which contained a stop codon was deleted and a Kozak translation initiation sequence was added. The resulting construct comprised 2088 bp of the FSH receptor coding region with a 5-base 5′ extension and a 92-base 3′ extension. The FSH receptor coding sequence with 5′ and 3′ extensions was excised by digestion with *Sma*I and *Kpn*I and blunt-end ligated into a blunted *EcoR*I site in the pSG5 vector (Stratagene).

A plasmid comprising the mutated receptor (*i.e.* the C640T allele) was created by site-directed mutagenesis of the wild-type cDNA. Site-directed mutagenesis was accomplished using a Clontech Transformer™ site-directed mutagenesis kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. The selection primer, 5′-GAGTGCACCATGGGCGGTGTGAAAT-3′ (SEQ ID NO:17), transformed an *Nde*I site into an *Nco*I site in the vector. The mutagenesis primer, 5′-GGGATTCAAGAAATACACAACTGTGTATTCAATGGAACCC-3′ (SEQ ID NO:18), accomplishes the C-to-T transition at position 640. Plasmid sequences were verified by restriction digestion and sequencing.

An MSC-1 cell line was used for transfection. That cell line was derived from a transgenic mouse Sertoli cell tumor generated by expressing the SV40 virus T-antigen under the control of the anti-muellerian hormone promoter reported in Peschon, *et al.*, *Mol. Endocrinol.*, *6*:1403-1411 (1992), incorporated by reference herein. Despite their Sertoli cell origin, MSC-1 cells do not express endogenous FSH receptor. MSC-1 cells in exponential growth phase were transiently transfected with either the wild-type or mutated FSH receptor-containing plasmids described above. Transfection was accomplished in DMEM/F12 (1:1) medium using lipofection (Gibco Life Technologies, Inc.) according to the manufacturer's instruction. A mock transfection with buffer was run as a control. Transfection efficiency was maintained by cotransfection with a luciferase-expressing pCmv-luci plasmid as reported in Gossen, *et al.*, *Proc. Nat'l. Acad. Sci.*

(USA), 89:5547-5551 (1992). Transfected cells were cultured in 2 cm culture plates.

Seventy-two hours after transfection, cells were exposed to one of 2, 10, 50, 100, or 200 IU/L of recombinant human FSH (rhFSH, Org 32489, approximately 10,000 IU/mg, Organon International BV). A control (vehicle only) was also run. After 3 hours, cells and media were removed from the culture plates and divided into two equal aliquots. One of the aliquots was diluted 1:1 with 2 mM theophylline, heated for 5 minutes at 100°C, spun for 5 minutes at 1500 g; and used to measure cAMP activity as described in Harper, *et al., J. Cycl. Nucleotide Res., 1*:207-218 (1975), incorporated by reference herein.

Stimulation with rhFSH of MSC-1 cells transfected with wild-type FSH receptor DNA produced a 3-4 fold dose-dependent stimulation of cAMP. The ED_{50} of stimulation was approximately 75 IU/L. In contrast, cells transfected with DNA comprising the C640T allele or mock-transfected with only pCmv-luci produced only negligible increases in cAMP activity, indicating that such cells are not stimulated by FSH. The results are shown in Figure 5, wherein squares denote transfections with the wild type allele, circles denote transfections with the mutant allele, and triangles represent mock transfections. Each data point in Figure 5 represents the mean of results in three identical experiments.

The foregoing results show that the ovarian dysgenesis allele (*i.e.* that with a substitution of T for C at position 640) results in the expression of a receptor which is unable to produce a signal upon stimulation by FSH.

EXAMPLE 6

MSC-1 cells which had been transfected with either mutant or wild-type FSH receptor plasmids were next used in FSH binding studies performed 48 hours after transfection. Cells were recovered and reconstituted to a concentration of $2x10^6$ cells/ml in buffer. The rhFSH described above was radiolabelled with ^{125}I iodine using the solid-phase lactoperoxidase method of Karonen, *et al., Anal. Biochem., 67*:1-10 (1975), incorporated by reference herein, to a specific activity of 30 Ci/g and 20% specific binding of radioactivity to an excess of FSH receptor as determined according to Catt, *et al., Methods in Receptor Research* (Belcher,

ed. 1976) 175-250, incorporated by reference herein. Triplicate aliquots of 100 μl each of the cell suspension (containing approximately 200,000 cells each) were incubated in the presence of 3.13, 6.25, 12.5, 25, 50, or 100 ng of radiolabelled rhFSH in a total volume of 250 μl. Non-specific binding was determined in the presence of 1.5 IU rhFSH. After overnight incubation at room temperature, radioactivity was measured in a gamma spectrometer.

Scatchard analysis revealed a 28-fold increase in binding of FSH to cells which had been transfected with wild-type FSH receptor-encoding DNA compared to cells transfected with DNA encoding the C640T allele. The equilibrium constant of FSH binding was $K_a=6.7 \times 10^9$ L/mol for the wild-type receptor and 4.8×10^9 L/mol for the mutated receptor. Results are shown in Figure 6, wherein circles represent wild-type receptors and triangles represent mutant receptors. In each case specific binding was equalized to a constant amount of luciferase activity. As shown in Figure 6, specific binding was 18.2 pmol/L for the wild type and 0.63 pmol/L for the ovarian dysgenesis allele.

The foregoing results show that a mutation from C to T at position 640 of the FSH receptor coding sequence is responsible for ovarian dysgenesis in human females and that because that mutation abolishes a *Bsm*I site, digestion with *Bsm*I or another restriction endonuclease which recognizes the *Bsm*I site is useful as a diagnostic tool for ovarian dysgenesis. It is apparent from the foregoing that other mutations may also produce the ovarian dysgenesis phenotype. Comparison of the nucleotide sequence of the FSH receptor gene in those cases with the wild-type sequence also provides a basis for diagnosis of the disease.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(F) POSTAL CODE: FIN-00710

(ii) TITLE OF INVENTION: Method For Diagnosis Of Ovarian Dysgenesis

(iii) NUMBER OF SEQUENCES: 18

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(E) COUNTRY: Finland
(F) POSTAL CODE: FIN-00120

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/531,070
(B) FILING DATE: 20-SEP-1995

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2179 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 75..2159

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGTGGAGCTT CTGAGATCTG TGGAGGTTTT TCTCTGCAAA TGCAGGAAGA AATCAGGTGG 60

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ATGGATGCAT AATT ATG GCC CTG CTC CTG GTC TCT TTG CTG GCA TTC CTG        110
                Met Ala Leu Leu Leu Val Ser Leu Leu Ala Phe Leu
                  1               5                  10

AGC TTG GGC TCA GGA TGT CAT CAT CGG ATC TGT CAC TGC TCT AAC AGG        158
Ser Leu Gly Ser Gly Cys His His Arg Ile Cys His Cys Ser Asn Arg
          15                  20                  25

GTT TTT CTC TGC CAA GAG AGC AAG GTG ACA GAG ATT CCT TCT GAC CTC        206
Val Phe Leu Cys Gln Glu Ser Lys Val Thr Glu Ile Pro Ser Asp Leu
     30                  35                  40

CCG AGG AAT GCC ATT GAA CTG AGG TTT GTC CTC ACC AAG CTT CGA GTC        254
Pro Arg Asn Ala Ile Glu Leu Arg Phe Val Leu Thr Lys Leu Arg Val
 45                  50                  55                  60

ATC CAA AAA GGT GCA TTT TCA GGA TTT GGG GAC CTG GAG AAA ATA GAG        302
Ile Gln Lys Gly Ala Phe Ser Gly Phe Gly Asp Leu Glu Lys Ile Glu
                65                  70                  75

ATC TCT CAG AAT GAT GTC TTG GAG GTG ATA GAG GCA GAT GTG TTC TCC        350
Ile Ser Gln Asn Asp Val Leu Glu Val Ile Glu Ala Asp Val Phe Ser
            80                  85                  90

AAC CTT CCC AAA TTA CAT GAA ATT AGA ATT GAA AAG GCC AAC AAC CTG        398
Asn Leu Pro Lys Leu His Glu Ile Arg Ile Glu Lys Ala Asn Asn Leu
          95                  100                 105

CTC TAC ATC AAC CCT GAG GCC TTC CAG AAC CTT CCC AAC CTT CAA TAT        446
Leu Tyr Ile Asn Pro Glu Ala Phe Gln Asn Leu Pro Asn Leu Gln Tyr
     110                 115                 120

CTG TTA ATA TCC AAC ACA GGT ATT AAG CAC CTT CCA GAT GTT CAC AAG        494
Leu Leu Ile Ser Asn Thr Gly Ile Lys His Leu Pro Asp Val His Lys
125                 130                 135                 140

ATT CAT TCT CTC CAA AAA GTT TTA CTT GAC ATT CAA GAT AAC ATA AAC        542
Ile His Ser Leu Gln Lys Val Leu Leu Asp Ile Gln Asp Asn Ile Asn
                145                 150                 155

ATC CAC ACA ATT GAA AGA AAT TCT TTC GTG GGG CTG AGC TTT GAA AGT        590
Ile His Thr Ile Glu Arg Asn Ser Phe Val Gly Leu Ser Phe Glu Ser
            160                 165                 170

GTG ATT CTA TGG CTG AAT AAG AAT GGG ATT CAA GAA ATA CAC AAC TGT        638
Val Ile Leu Trp Leu Asn Lys Asn Gly Ile Gln Glu Ile His Asn Cys
          175                 180                 185

GCA TTC AAT GGA ACC CAA CTA GAT GAG CTG AAT CTA AGC GAT AAT AAT        686
Ala Phe Asn Gly Thr Gln Leu Asp Glu Leu Asn Leu Ser Asp Asn Asn
     190                 195                 200

AAT TTA GAA GAA TTG CCT AAT GAT GTT TTC CAC GGA GCC TCT GGA CCA        734
Asn Leu Glu Glu Leu Pro Asn Asp Val Phe His Gly Ala Ser Gly Pro
205                 210                 215                 220

GTC ATT CTA GAT ATT TCA AGA ACA AGG ATC CAT TCC CTG CCT AGC TAT        782
Val Ile Leu Asp Ile Ser Arg Thr Arg Ile His Ser Leu Pro Ser Tyr
                225                 230                 235

GGC TTA GAA AAT CTT AAG AAG CTG AGG GCC AGG TCG ACT TAC AAC TTA        830
Gly Leu Glu Asn Leu Lys Lys Leu Arg Ala Arg Ser Thr Tyr Asn Leu
            240                 245                 250
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AAA AAG CTG CCT ACT CTG GAA AAG CTT GTC GCC CTC ATG GAA GCC AGC          878
Lys Lys Leu Pro Thr Leu Glu Lys Leu Val Ala Leu Met Glu Ala Ser
        255                 260                 265

CTC ACC TAT CCC AGC CAT TGC TGT GCC TTT GCA AAC TGG AGA CGG CAA          926
Leu Thr Tyr Pro Ser His Cys Cys Ala Phe Ala Asn Trp Arg Arg Gln
    270                 275                 280

ATC TCT GAG CTT CAT CCA ATT TGC AAC AAA TCT ATT TTA AGG CAA GAA          974
Ile Ser Glu Leu His Pro Ile Cys Asn Lys Ser Ile Leu Arg Gln Glu
285                 290                 295                 300

GTT GAT TAT ATG ACT CAG ACT AGG GGT CAG AGA TCC TCT CTG GCA GAA         1022
Val Asp Tyr Met Thr Gln Thr Arg Gly Gln Arg Ser Ser Leu Ala Glu
                305                 310                 315

GAC AAT GAG TCC AGC TAC AGC AGA GGA TTT GAC ATG ACG TAC ACT GAG         1070
Asp Asn Glu Ser Ser Tyr Ser Arg Gly Phe Asp Met Thr Tyr Thr Glu
            320                 325                 330

TTT GAC TAT GAC TTA TGC AAT GAA GTG GTT GAC GTG ACC TGC TCC CCT         1118
Phe Asp Tyr Asp Leu Cys Asn Glu Val Val Asp Val Thr Cys Ser Pro
        335                 340                 345

AAG CCA GAT GCA TTC AAC CCA TGT GAA GAT ATC ATG GGG TAC AAC ATC         1166
Lys Pro Asp Ala Phe Asn Pro Cys Glu Asp Ile Met Gly Tyr Asn Ile
    350                 355                 360

CTC AGA GTC CTG ATA TGG TTT ATC AGC ATC CTG GCC ATC ACT GGG AAC         1214
Leu Arg Val Leu Ile Trp Phe Ile Ser Ile Leu Ala Ile Thr Gly Asn
365                 370                 375                 380

ATC ATA GTG CTA GTG ATC CTA ACT ACC AGC CAA TAT AAA CTC ACA GTC         1262
Ile Ile Val Leu Val Ile Leu Thr Thr Ser Gln Tyr Lys Leu Thr Val
                385                 390                 395

CCC AGG TTC CTT ATG TGC AAC CTG GCC TTT GCT GAT CTC TGC ATT GGA         1310
Pro Arg Phe Leu Met Cys Asn Leu Ala Phe Ala Asp Leu Cys Ile Gly
            400                 405                 410

ATC TAC CTG CTG CTC ATT GCA TCA GTT GAT ATC CAT ACC AAG AGC CAA         1358
Ile Tyr Leu Leu Leu Ile Ala Ser Val Asp Ile His Thr Lys Ser Gln
        415                 420                 425

TAT CAC AAC TAT GCC ATT GAC TGG CAA ACT GGG GCA GGC TGT GAT GCT         1406
Tyr His Asn Tyr Ala Ile Asp Trp Gln Thr Gly Ala Gly Cys Asp Ala
    430                 435                 440

GCT GGC TTT TTC ACT GTC TTT GCC AGT GAG CTG TCA GTC TAC ACT CTG         1454
Ala Gly Phe Phe Thr Val Phe Ala Ser Glu Leu Ser Val Tyr Thr Leu
445                 450                 455                 460

ACA GCT ATC ACC TTG GAA AGA TGG CAT ACC ATC ACG CAT GCC ATG CAG         1502
Thr Ala Ile Thr Leu Glu Arg Trp His Thr Ile Thr His Ala Met Gln
                465                 470                 475

CTG GAC TGC AAG GTG CAG CTC CGC CAT GCT GCC AGT GTC ATG GTG ATG         1550
Leu Asp Cys Lys Val Gln Leu Arg His Ala Ala Ser Val Met Val Met
            480                 485                 490

GGC TGG ATT TTT GCT TTT GCA GCT GCC CTC TTT CCC ATC TTT GGC ATC         1598
Gly Trp Ile Phe Ala Phe Ala Ala Ala Leu Phe Pro Ile Phe Gly Ile
        495                 500                 505
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AGC AGC TAC ATG AAG GTG AGC ATC TGC CTG CCC ATG GAT ATT GAC AGC      1646
Ser Ser Tyr Met Lys Val Ser Ile Cys Leu Pro Met Asp Ile Asp Ser
    510                 515                 520

CCT TTG TCA CAG CTG TAT GTC ATG TCC CTC CTT GTG CTC AAT GTC CTG      1694
Pro Leu Ser Gln Leu Tyr Val Met Ser Leu Leu Val Leu Asn Val Leu
525                 530                 535                 540

GCC TTT GTG GTC ATC TGT GGC TGC TAT ATC CAC ATC TAC CTC ACA GTG      1742
Ala Phe Val Val Ile Cys Gly Cys Tyr Ile His Ile Tyr Leu Thr Val
                545                 550                 555

CGG AAC CCC AAC ATC GTG TCC TCC TCT AGT GAC ACC AGG ATC GCC AAG      1790
Arg Asn Pro Asn Ile Val Ser Ser Ser Ser Asp Thr Arg Ile Ala Lys
            560                 565                 570

CGC ATG GCC ATG CTC ATC TTC ACT GAC TTC CTC TGC ATG GCA CCC ATT      1838
Arg Met Ala Met Leu Ile Phe Thr Asp Phe Leu Cys Met Ala Pro Ile
        575                 580                 585

TCT TTC TTT GCC ATT TCT GCC TCC CTC AAG GTG CCC CTC ATC ACT GTG      1886
Ser Phe Phe Ala Ile Ser Ala Ser Leu Lys Val Pro Leu Ile Thr Val
    590                 595                 600

TCC AAA GCA AAG ATT CTG CTG GTT CTG TTT CAC CCC ATC AAC TCC TGT      1934
Ser Lys Ala Lys Ile Leu Leu Val Leu Phe His Pro Ile Asn Ser Cys
605                 610                 615                 620

GCC AAC CCC TTC CTC TAT GCC ATC TTT ACC AAA AAC TTT CGC AGA GAT      1982
Ala Asn Pro Phe Leu Tyr Ala Ile Phe Thr Lys Asn Phe Arg Arg Asp
                625                 630                 635

TTC TTC ATT CTG CTG AGC AAG TGT GGC TGC TAT GAA ATG CAA GCC CAA      2030
Phe Phe Ile Leu Leu Ser Lys Cys Gly Cys Tyr Glu Met Gln Ala Gln
            640                 645                 650

ATT TAT AGG ACA GAA ACT TCA TCC ACT GTC CAC AAC ACC CAT CCA AGG      2078
Ile Tyr Arg Thr Glu Thr Ser Ser Thr Val His Asn Thr His Pro Arg
        655                 660                 665

AAT GGC CAC TGC TCT TCA GCT CCC AGA GTC ACC AAT GGT TCC ACT TAC      2126
Asn Gly His Cys Ser Ser Ala Pro Arg Val Thr Asn Gly Ser Thr Tyr
    670                 675                 680

ATA CTT GTC CCT CTA AGT CAT TTA GCC CAA AAC TAAAACACAA TGTGAAAATG    2179
Ile Leu Val Pro Leu Ser His Leu Ala Gln Asn
685                 690                 695
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CGCCCGCCGC GCCCCGCGCC CGGCCCGCCG CCCCCGCCCG GACTTATGCA ATGAAGTGGT      60

TG                                                                     62
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTGAAAAAGC CAGCAGCATC 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGCCCGCCGC GCCCCGCGCC CGGCCCGCCG CCCCCGCCCG ATTGACTGGC AAACTGGGG 59

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGAGGAGGAC ACGATGTTGG 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCCCGCCGC GCCCCGCGCC CGGCCCGCCG CCCCCGCCCG GGCTGCTATA TCCACATCTA 60

CC 62

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAGAACCAGC AGAATCTTTG C 21

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGCCCGCCGC GCCCCGCGCC CGGCCCGCCG CCCCCGCCCG CTTTCTTTGC CATTTCTGCC 60

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CAAAGGCAAG GACTGAATTA TC 22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGCAAAGATT CTGCTGGTTC 20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TAGTTTTGGG CTAAATGACT TAGAGGG 27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCTGCTCCTG GTCTCTTTGC TG 22

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGAAATTCTT TCGTGGGGCT 20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTTGCAAAG GCACAGCAAT 20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTTATTTCAG ATGGCTGAAT AAG 23

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCTCATCTAG TTGGGTTC 18

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAGTGCACCA TGGGCGGTGT GAAAT 25

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGGATTCAAG AAATACACAA CTGTGTATTC AATGGAACCC 40

CLAIMS

We claim:

1. A method for determining a follicle-stimulating hormone receptor (*fshr*) genotype in a human patient, comprising the steps of:

(a) providing a biological sample comprising nucleic acid from said patient, said nucleic acid including said patient's *fshr* alleles;

(b) analyzing said nucleic acid for the presence of a mutation or mutations in codon 189 of said *fshr* alleles; and

(c) determining an *fshr* genotype from said analyzing step, wherein the presence of a mutation in codon 189 of a *fshr* allele is correlated with an ovarian dysgenesis genotype.

2. The method according to claim 1 wherein said biological sample is a cell sample.

3. The method according to claim 1 or 2 wherein said patient is a female.

4. The method according to any one of claims 1-3 wherein said analyzing comprises sequencing a portion of said nucleic acid, said portion comprising codon 189 of said *fshr* alleles.

5. The method according to any one of claims 1-4 wherein said nucleic acid is DNA.

6. The method according to claim 5 wherein said analyzing step comprises the steps of:

(a) exposing said nucleic acid to a restriction endonuclease having a recognition site that includes codon 189 of a wild type *fshr* allele, under conditions wherein said restriction endonuclease specifically cleaves DNA at its recognition site; and

(b) detecting said nucleic acid or polynucleotide restriction fragments thereof from said exposing step.

7. The method according to claim 6 wherein said restriction endonuclease is selected from the group consisting of *Bsm*I, restriction endonucleases recognizing sites that overlap a *Bsm*I site, restriction endonucleases recognizing the same cleavage sites as *Bsm*I, and isoschizomers of *Bsm*I.

8. The method according to claim 6 wherein said restriction endonuclease is *Bsm*I.

9. A method for screening for an ovarian dysgenesis genotype in a patient, comprising the steps of:

(a) providing a biological sample comprising nucleic acid from said patient, said nucleic acid including said patient's follicle-stimulating hormone receptor (*fshr*) alleles;

(b) amplifying a portion of said nucleic acid to generate amplified DNA, said portion including codon 189 of said *fshr* alleles;

(c) exposing said amplified DNA to a restriction endonuclease selected from the group consisting of *Bsm*I restriction endonuclease and restriction endonucleases having a recognition site which overlaps a *Bsm*I recognition site, under conditions wherein said restriction endonuclease specifically cleaves DNA at the recognition site of said restriction endonuclease;

(d) thereafter detecting said amplified DNA or restriction fragments thereof;

(e) comparing the amplified DNA or fragments thereof of step (d) to control nucleic acid of a human subject free of an ovarian dysgenesis genotype, wherein said control nucleic acid has been amplified, exposed, and detected in accordance with steps (b), (c), and (d); and

(f) screening for an ovarian dysgenesis genotype from said comparison, wherein an ovarian dysgenesis genotype in said patient is correlated with a

different number of detected amplified DNA or restriction fragments thereof from said patient's amplified DNA than from said control amplified DNA.

10. The method according to claim 9 wherein in said screening step a homozygous ovarian dysgenesis genotype is correlated with fewer detected restriction fragments from said patient's amplified DNA than from said control amplified DNA.

11. The method according to claim 9 wherein in said screening step a heterozygous ovarian dysgenesis genotype is correlated with a greater number of detected amplified DNA or restriction fragments thereof from said patient's amplified DNA than from said control amplified DNA.

12. The method according to claim 9 wherein said patient is a human female.

13. The method according to claim 9 wherein said portion comprises exon 7 of said *fshr* alleles.

14. A method for diagnosing ovarian dysgenesis in a female patient, comprising the steps of:

obtaining a cell sample from a female patient;

isolating nucleic acids from said cell sample;

amplifying a portion of said nucleic acids encoding a receptor for follicle-stimulating hormone, thereby generating amplified DNA;

exposing said amplified DNA to a restriction endonuclease selected from the group consisting of *Bsm*I and restriction endonucleases having a recognition site which overlaps that of *Bsm*I, under conditions wherein said restriction endonuclease specifically cleaves DNA at its recognition site;

detecting polynucleotide restriction fragments of said amplified DNA; and

diagnosing ovarian dysgenesis from said restriction fragments, wherein ovarian dysgenesis is correlated with a C to T mutation in codon 189 of said DNA encoding a receptor for follicle-stimulating hormone, said mutation eliminating a recognition site of said restriction endonuclease.

15. A method for diagnosing ovarian dysgenesis in a female patient comprising the steps of:

obtaining a cell sample from a female patient;

isolating nucleic acids from said cell sample;

amplifying a portion of said nucleic acids comprising exon 7 of a follicle-stimulating hormone receptor gene, said portion excluding any *Bsm*I site outside of exon 7, thereby generating amplified DNA;

exposing said amplified DNA to a restriction endonuclease selected from the group consisting of *Bsm*I and restriction endonucleases having a recognition site which overlaps that of *Bsm*I, under conditions wherein said restriction endonuclease specifically cleaves DNA at its recognition site;

detecting said amplified DNA or polynucleotide restriction fragments thereof after said exposing step; and

diagnosing ovarian dysgenesis from said detecting step, wherein ovarian dysgenesis is correlated with the detection of said amplified DNA and the absence of polynucleotide restriction fragments thereof.

16. The method according to claim 14 or 15, wherein said restriction endonucleases having a recognition site which overlaps that of *Bsm*I are selected from the group consisting of *Sac*III and *Sst*III.

17. A method for diagnosing ovarian dysgenesis in a female patient suspected of having ovarian dysgenesis, comprising the steps of:

obtaining a cell sample from said female patient;

isolating nucleic acid from said cell sample;

amplifying a portion of said isolated nucleic acid comprising exon 7 of a follicle-stimulating hormone receptor gene, thereby generating amplified DNA;

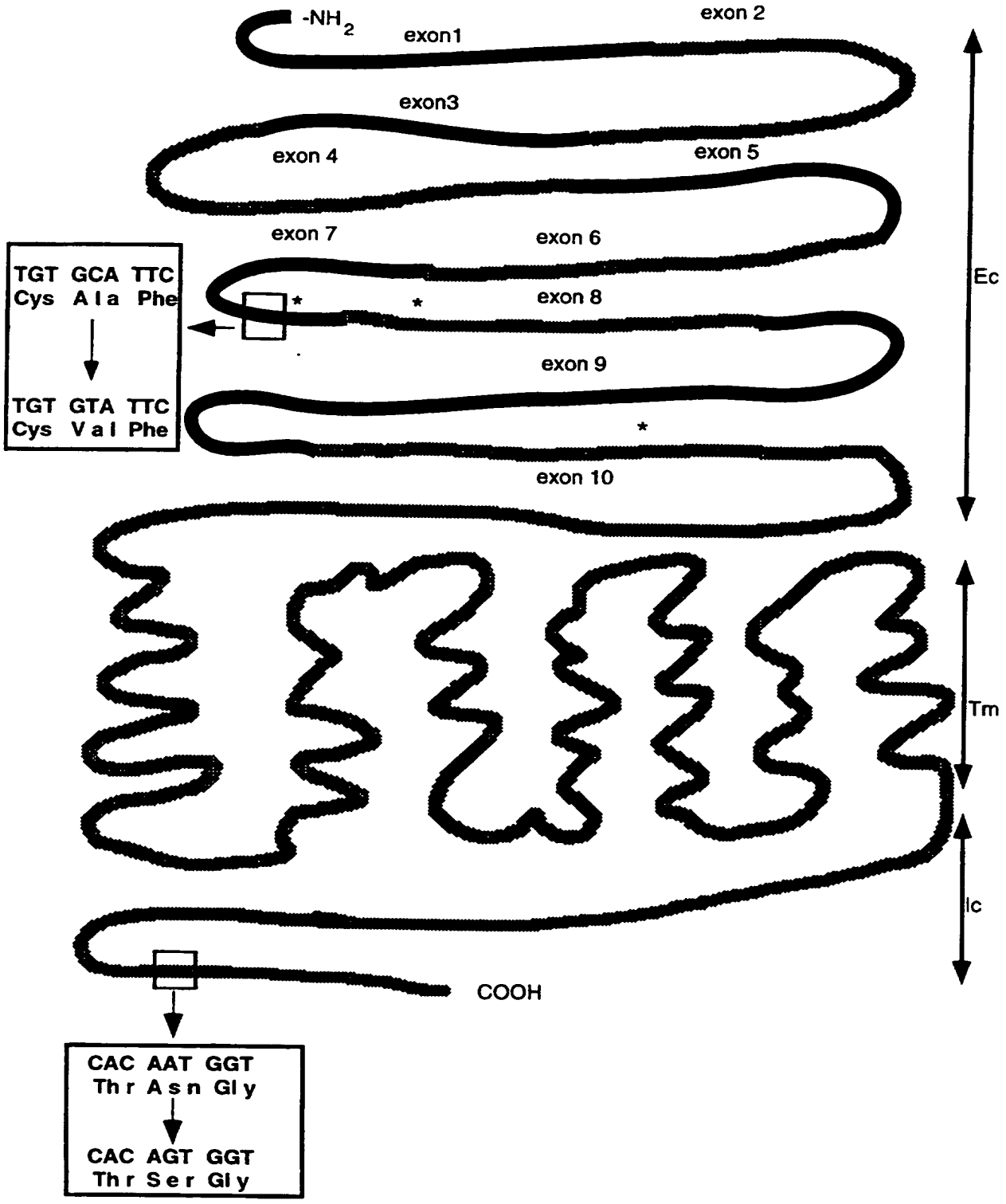
sequencing said amplified DNA;

comparing the sequence of said amplified DNA to SEQ ID NO:1; and

diagnosing ovarian dysgenesis by the presence of one or more sequence differences between the sequence of said amplified DNA and SEQ ID NO:1.

18. The method according to claim 17, wherein said one or more differences between the sequence of said amplified DNA and SEQ ID NO:1 comprises a difference at nucleotide position 640 in SEQ ID NO:1.

19. The method according to claim 18, wherein a cytidylate at position 640 in SEQ ID NO:1 is a thymidylate at a corresponding position in said amplified DNA.



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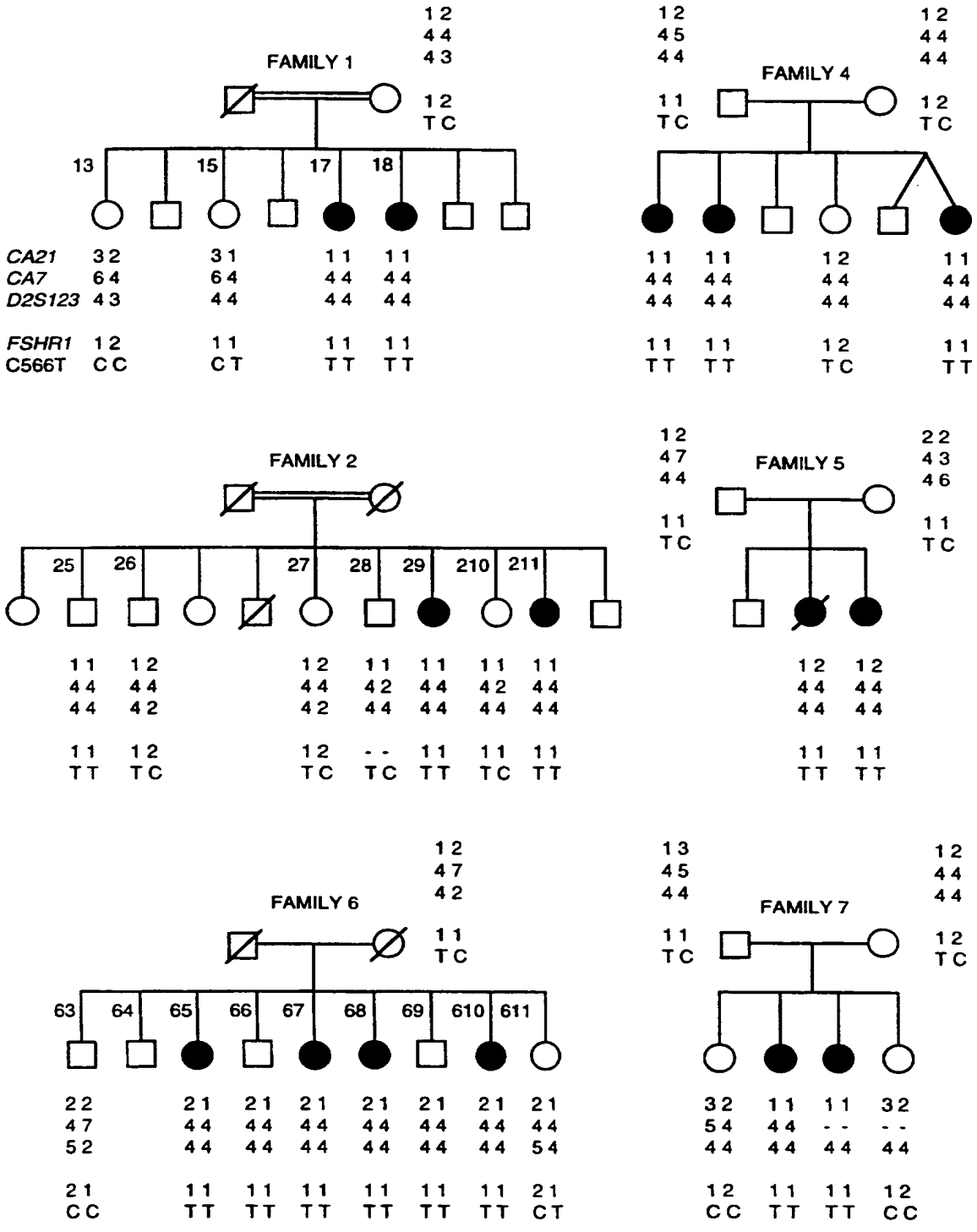
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FAMILY 6
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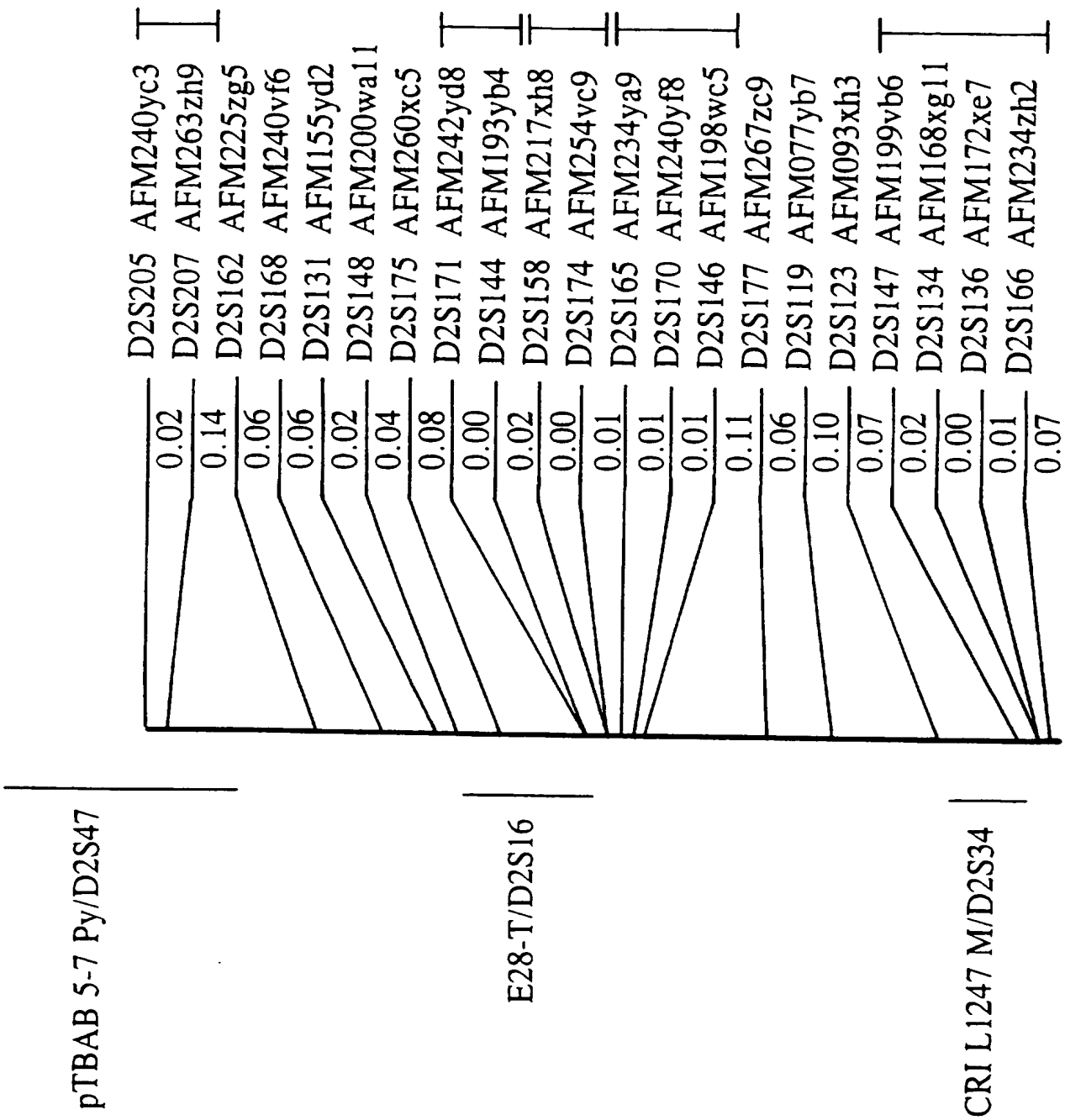
FIG. 2



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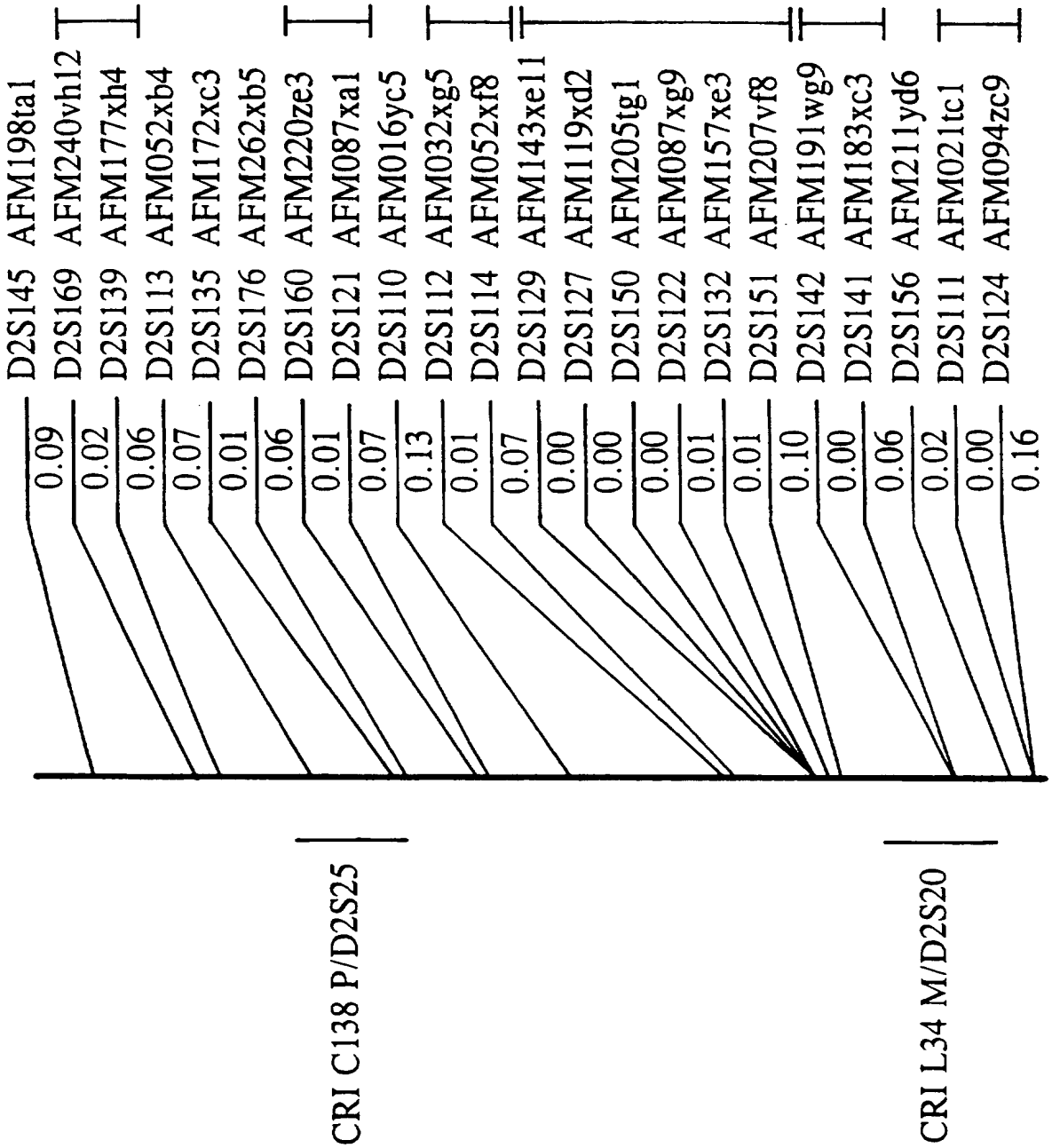

FIG. 3 (2of 4)

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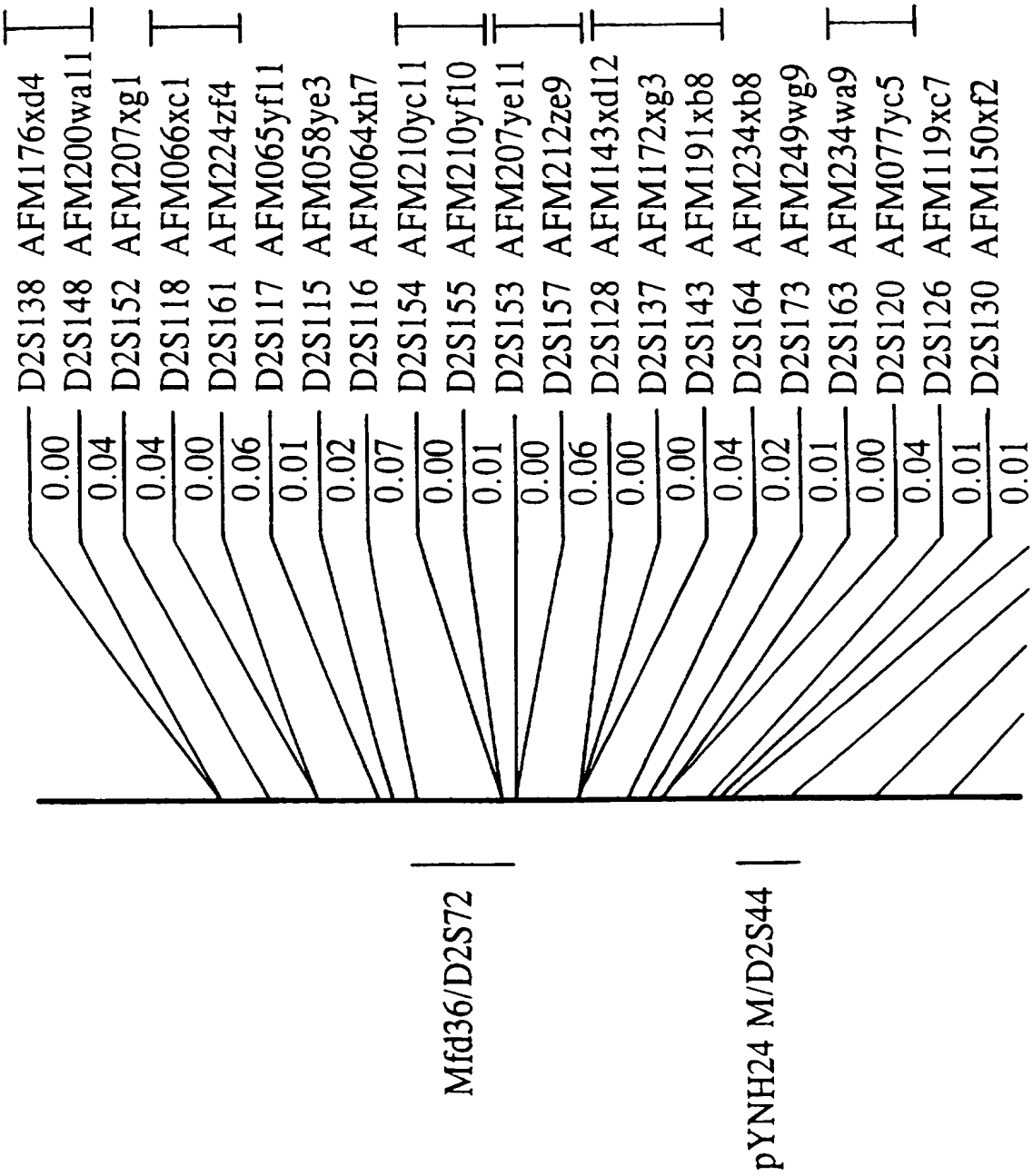
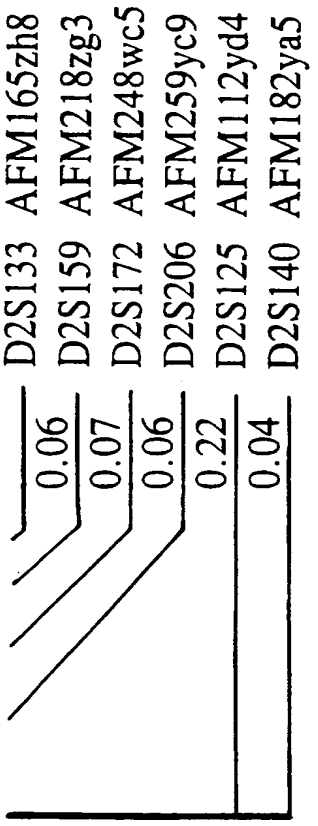


FIG. 3 (3of 4)

FIG. 3 (4of 4)



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Ser Leu Gly Ser Gly Cys His His Arg Ile Cys His Cys Ser Asn Arg       28

GTT TTT CTC TGC CAA GAG AGC AAG GTG ACA GAG ATT CCT TCT GAC CTC      206
Val Phe Leu Cys Gln Glu Ser Lys Val Thr Glu Ile Pro Ser Asp Leu       44

CCG AGG AAT GCC ATT GAA CTG AGG TTT GTC CTC ACC AAG CTT CGA GTC      254
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FIG. 4 (1 of 2)

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FIG. 4 (2 of 2)

FIG. 5

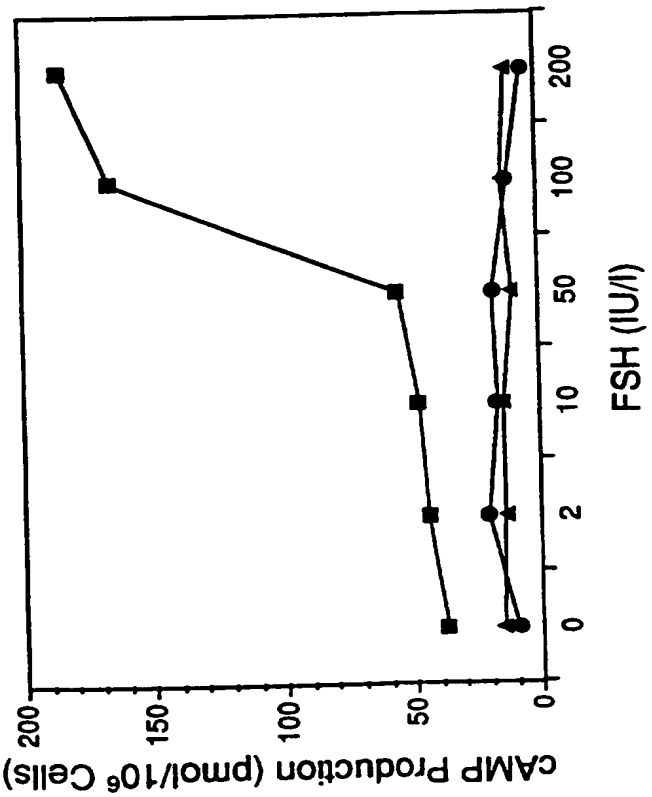
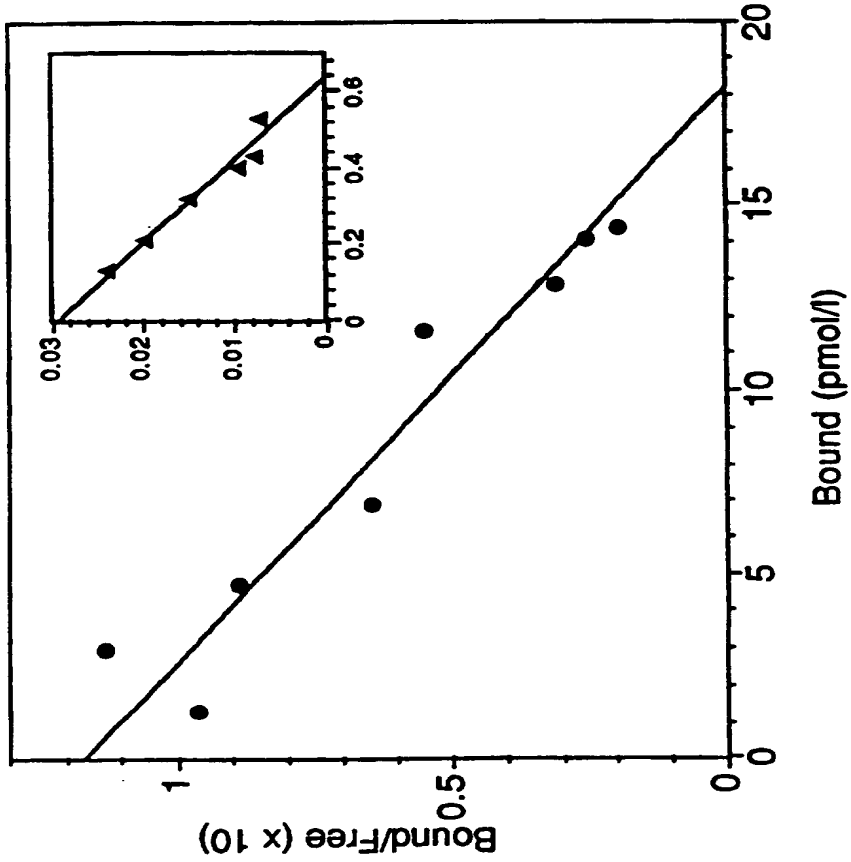


FIG. 6



INTERNATIONAL SEARCH REPORT

International Application No
PCT/FI 96/00501

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	CELL, vol. 82, no. 6, 22 September 1995, pages 959-68, XP000609143 AITTOMÄKI, K. ET AL: "Mutation in the follicle-stimulating hormone receptor causes heriditary hypergonadotropic ovarian failure " see the whole document ---	1-18
A	AMERICAN JOURNAL OF HUMAN GENETICS, vol. 54, no. 5, May 1994, pages 844-51, XP000612836 AITTOMÄKI, K.: "The genetics of XX gonadal dysgenesis" cited in the application see the whole document ---	1

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
11 December 1996	07. 01. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

Inter ial Application No
PCT/FI 96/00501

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	NATURE GENETICS, vol. 9, February 1995, pages 160-4, XP000612866 KREMER H ET AL: "Male pseudohermaphroditism due to a homzygous missense mutation of the luteinizing hormone receptor gene" see the whole document ---	1
A	GENOMICS , vol. 15, January 1993, pages 222-24, XP000612862 ROUSSEAU-MERCK M ET AL: "The chromosomal localization of the human follicle hormone receptor gene (FSHR) on 2p21-2p15 is similar to that of the luteinizing hormone receptor gene" cited in the application see the whole document -----	1

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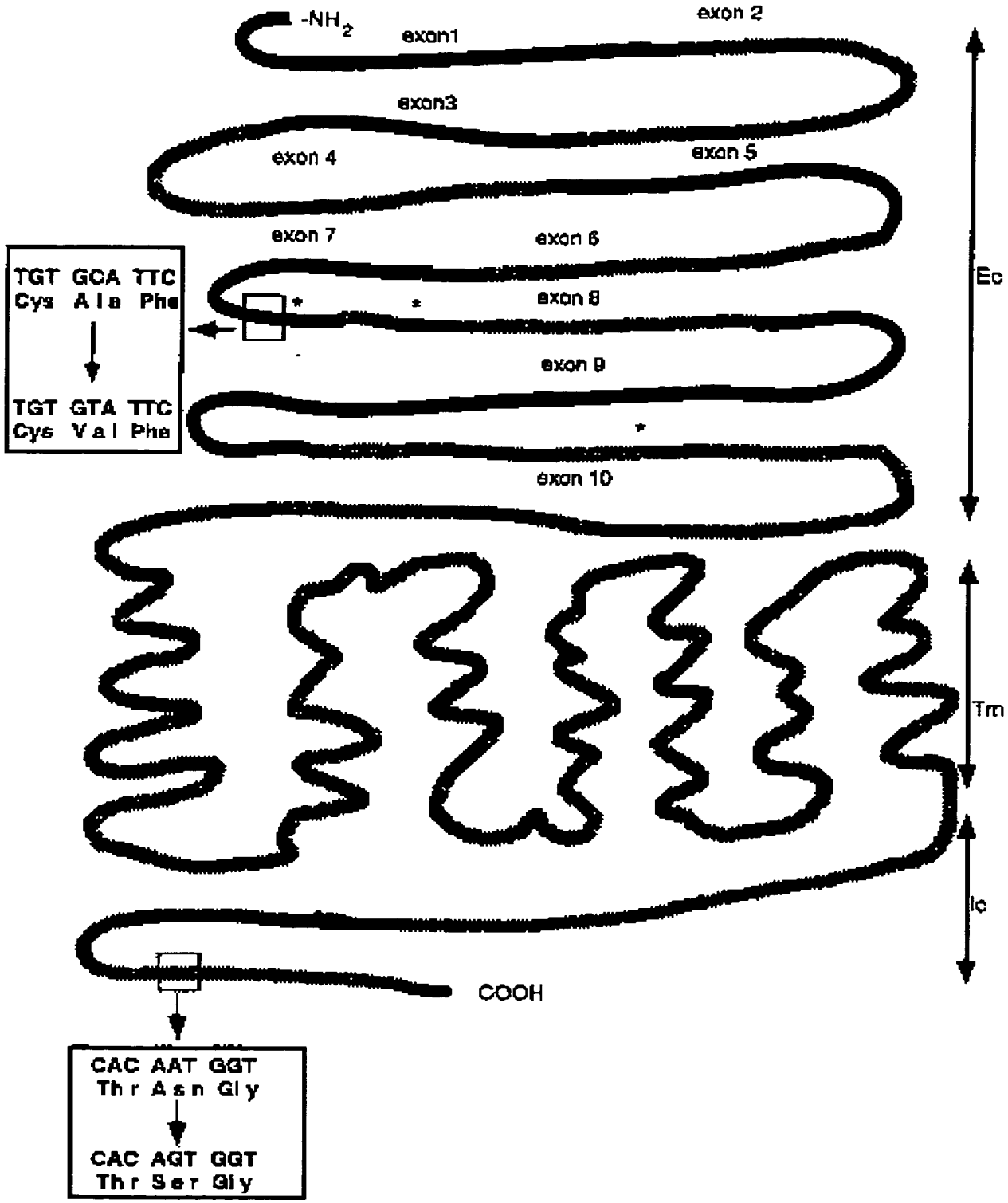

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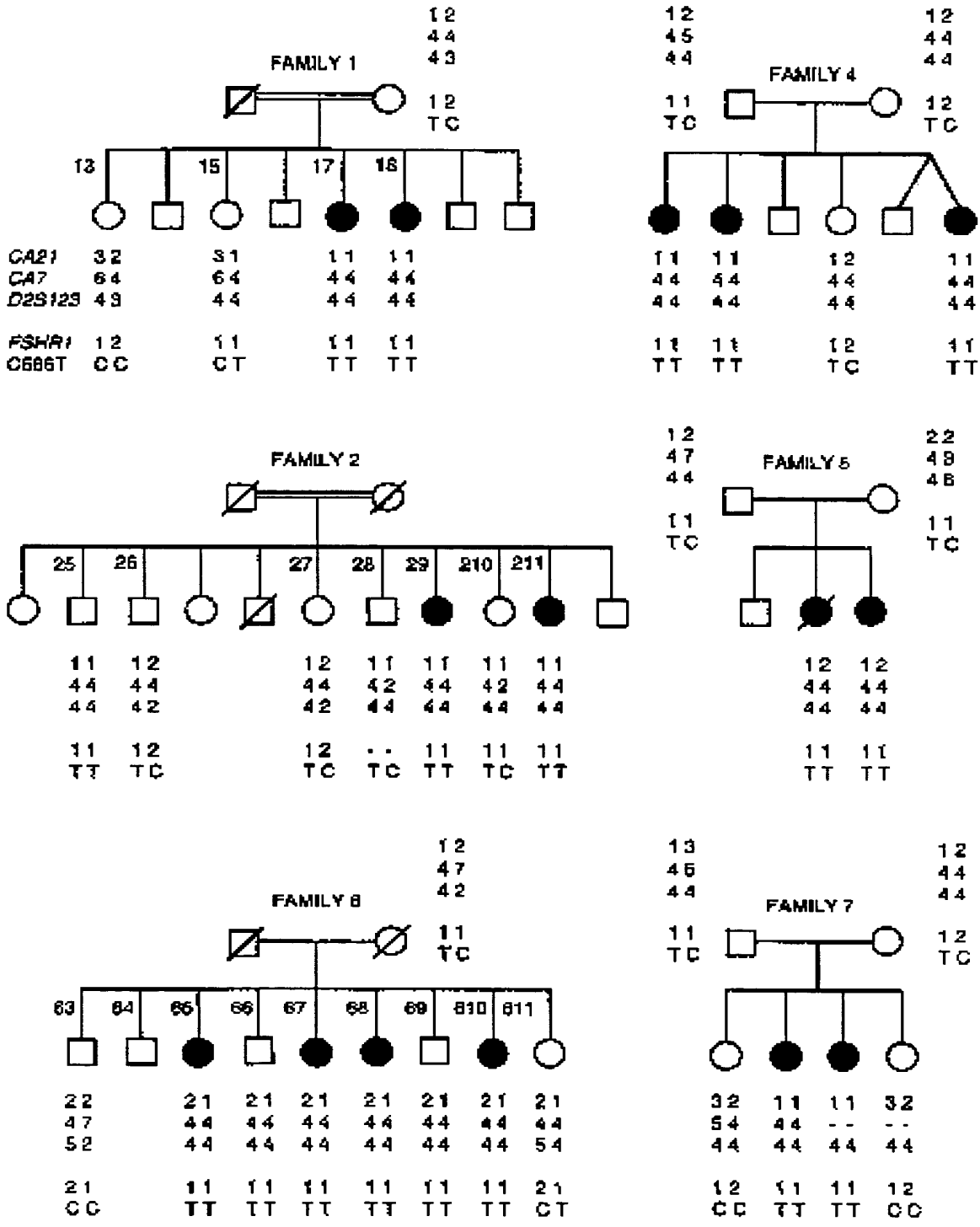
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FIG. 2

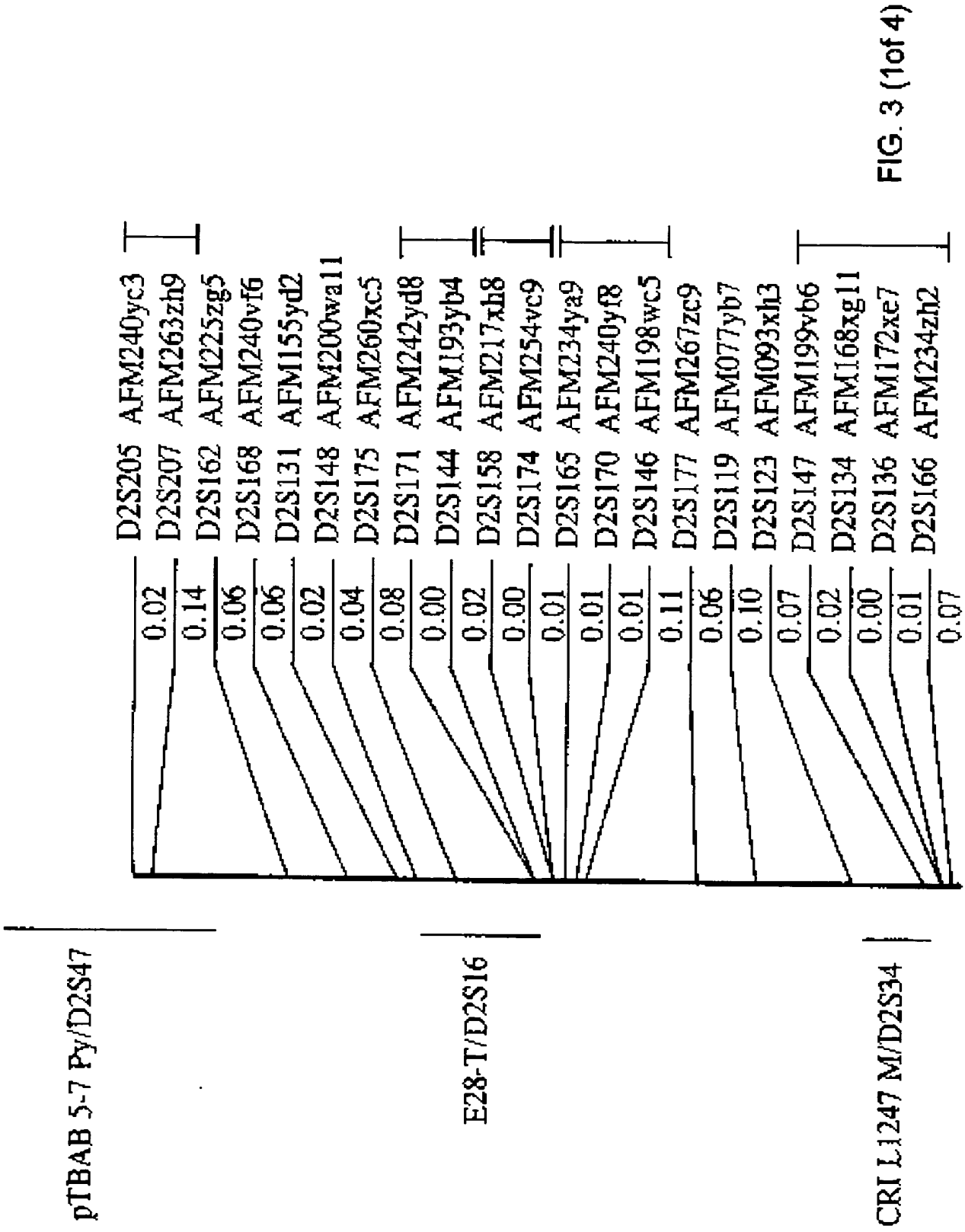
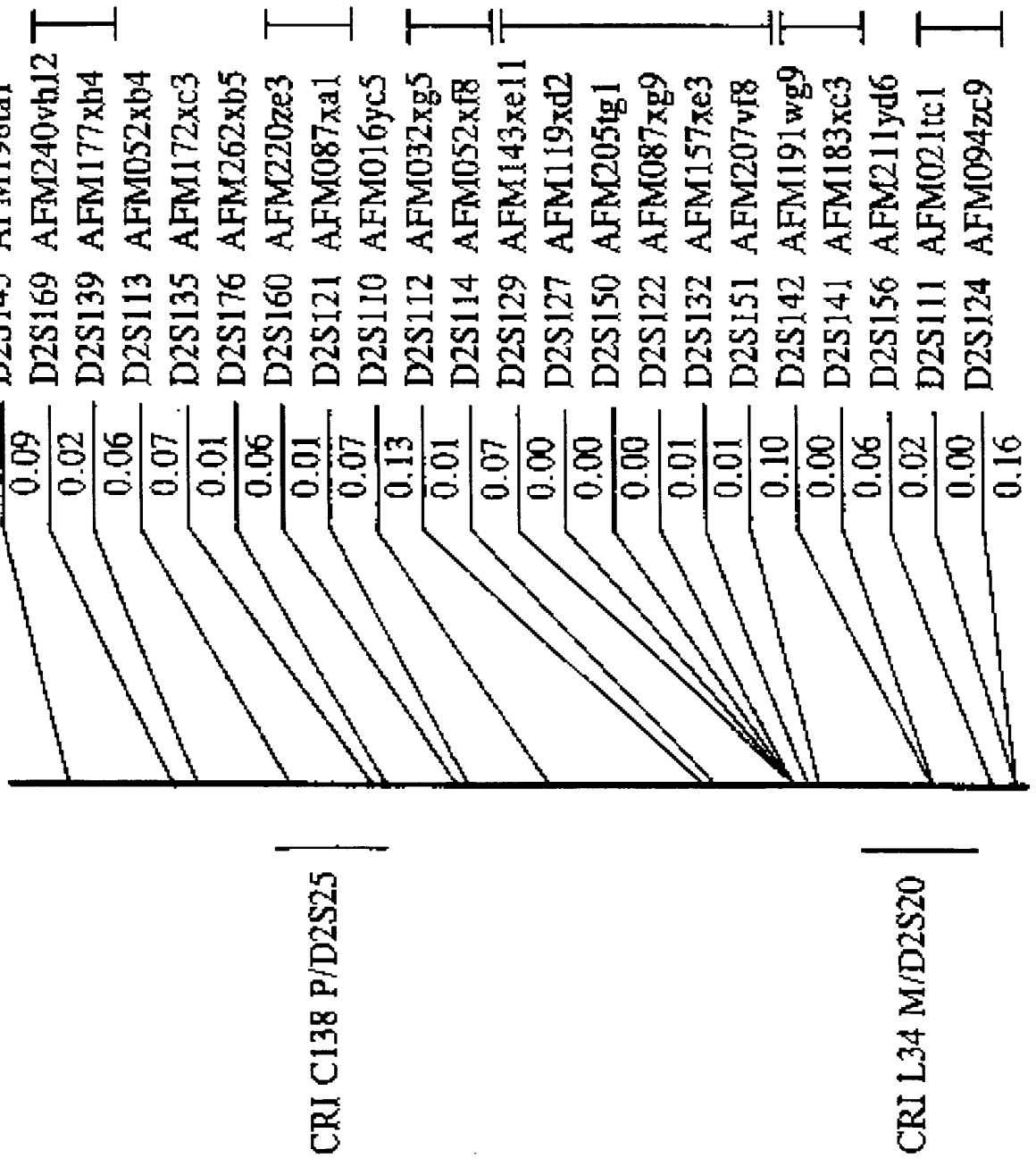


FIG. 3 (1of 4)



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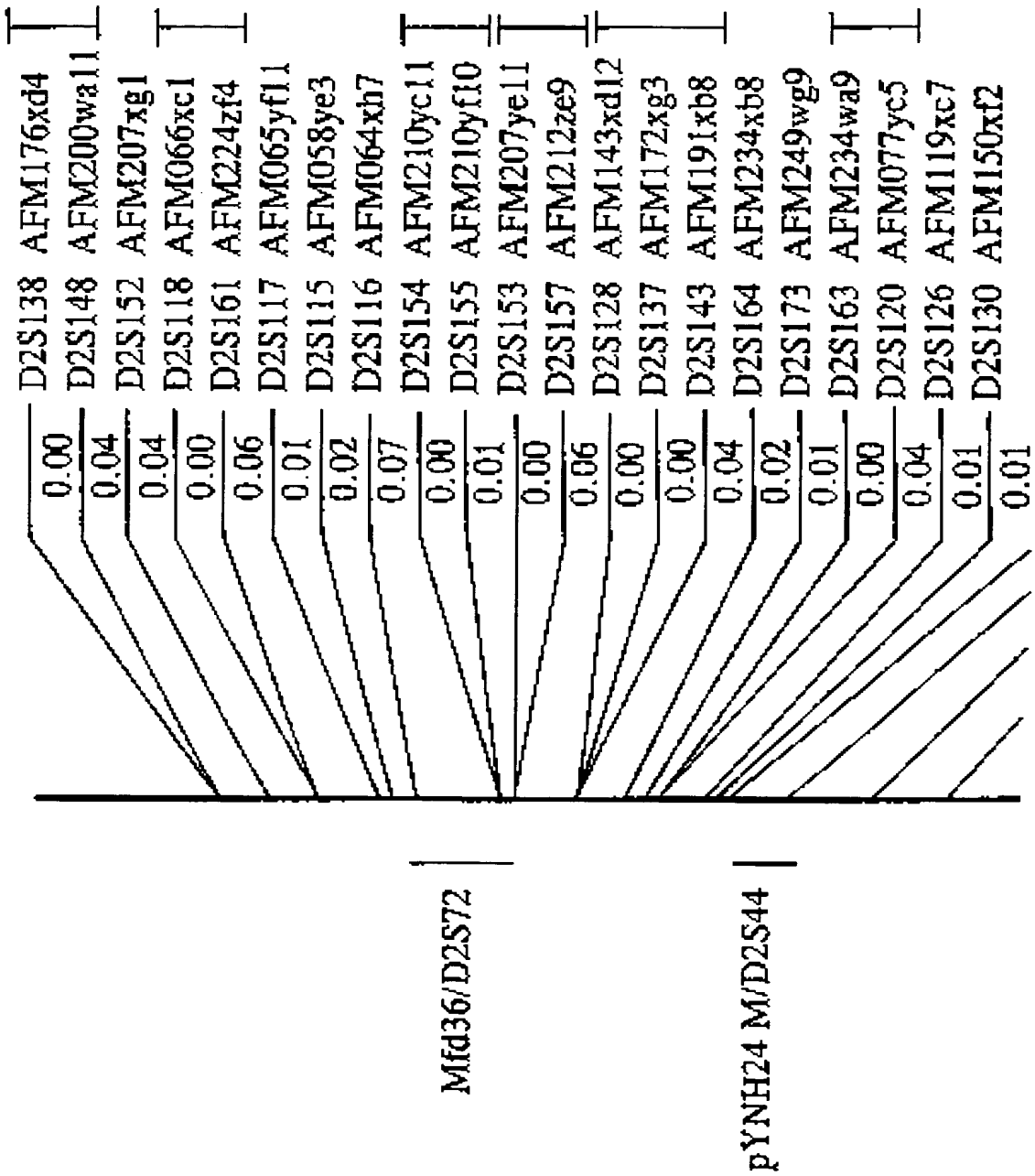
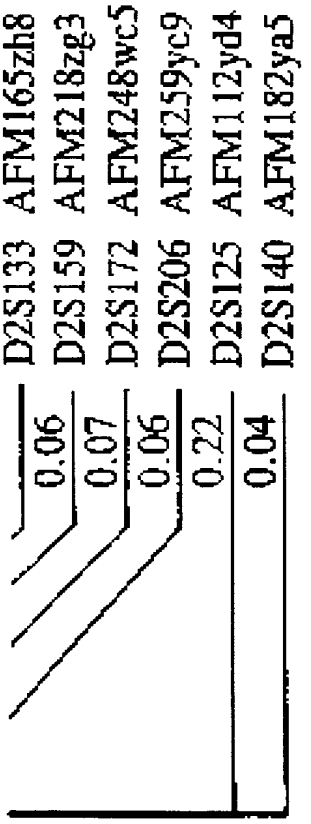


FIG. 3 (3of 4)

FIG. 3 (4of 4)



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Val Phe Leu Cys Gln Glu Ser Lys Val Thr Glu Ile Pro Ser Asp Leu       44

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Pro Arg Asn Ala Ile Glu Leu Arg Phe Val Leu Thr Lys Leu Arg Val       60

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Ile Gln Lys Gly Ala Phe Ser Gly Phe Gly Asp Leu Glu Lys Ile Glu       76

ATC TCT CAG AAT GAT GTC TTG GAG GTG ATA GAG GCA GAT GTG TTC TCC      350
Ile Ser Gln Asn Asp Val Leu Glu Val Ile Glu Ala Asp Val Phe Ser       92

AAC CTT CCC AAA TTA CAT GAA ATT AGA ATT GAA AAG GCC AAC AAC CTG      398
Asn Leu Pro Lys Leu His Glu Ile Arg Ile Glu Lys Ala Asn Asn Leu      108

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Leu Tyr Ile Asn Pro Glu Ala Phe Gln Asn Leu Pro Asn Leu Gln Tyr      124

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Leu Leu Ile Ser Asn Thr Gly Ile Lys His Leu Pro Asp Val His Lys      140

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Ile His Ser Leu Gln Lys Val Leu Leu Asp Ile Gln Asp Asn Ile Asn      156

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Ile His Thr Ile Glu Arg Asn Ser Phe Val Gly Leu Ser Phe Glu Ser      172

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GCA TTC AAT GGA ACC CAA CTA GAT GAG CTG AAT CTA AGC GAT AAT AAT      686
Ala Phe Asn Gly Thr Gln Leu Asp Glu Leu Asn Leu Ser Asp Asn Asn      204

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Asn Leu Glu Glu Leu Pro Asn Asp Val Phe His Gly Ala Ser Gly Pro      220

GTC ATT CTA GAT ATT TCA AGA ACA AGG ATC CAT TCC CTG CCT AGC TAT      782
Val Ile Leu Asp Ile Ser Arg Thr Arg Ile His Ser Leu Pro Ser Tyr      236

GGC TTA GAA AAT CTT AAG AAG CTG AGG GCC AGG TCG ACT TAC AAC TTA      830
Gly Leu Glu Asn Leu Lys Lys Leu Arg Ala Arg Ser Thr Tyr Asn Leu      252

AAA AAG CTG CCT ACT CTG GAA AAG CTT GTC GCC CTC ATG GAA GCC AGC      878
Lys Lys Leu Pro Thr Leu Glu Lys Leu Val Ala Leu Met Glu Ala Ser      268

CTC ACC TAT CCC AGC CAT TGC TGT GCC TTT GCA AAC TGG AGA CGG CAA      926
Leu Thr Tyr Pro Ser His Cys Cys Ala Phe Ala Asn Trp Arg Arg Gln      284

ATC TCT GAG CTT CAT CCA ATT TGC AAC AAA TCT ATT TTA AGG CAA GAA      974
Ile Ser Glu Leu His Pro Ile Cys Asn Lys Ser Ile Leu Arg Gln Glu      300

GTT GAT TAT ATG ACT CAG ACT AGG GGT CAG AGA TCC TCT CTG GCA GAA     1022
Val Asp Tyr Met Thr Gln Thr Arg Gly Gln Arg Ser Ser Leu Ala Glu      316

GAC AAT GAG TCC AGC TAC AGC AGA GGA TTT GAC ATG ACG TAC ACT GAG     1070
Asp Asn Glu Ser Ser Tyr Ser Arg Gly Phe Asp Met Thr Tyr Thr Glu      332

TTT GAC TAT GAC TTA TGC AAT GAA GTG GTT GAC GTG ACC TGC TCC CCT     1118
Phe Asp Tyr Asp Leu Cys Asn Glu Val Val Asp Val Thr Cys Ser Pro      348
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FIG. 4 (1 of 2)

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AAG CCA GAT GCA TTC AAC CCA TGT GAA GAT ATC ATG GGG TAC AAC ATC     1166
Lys Pro Asp Ala Phe Asn Pro Cys Glu Asp Ile Met Gly Tyr Asn Ile      364

CTC AGA GTC CTG ATA TGG TTT ATC AGC ATC CTG GCC ATC ACT GGG AAC     1214
Leu Arg Val Leu Ile Trp Phe Ile Ser Ile Leu Ala Ile Thr Gly Asn      380

ATC ATA GTG CTA GTG ATC CTA ACT ACC AGC CAA TAT AAA CTC ACA GTC     1262
Ile Ile Val Leu Val Ile Leu Thr Thr Ser Gln Tyr Lys Leu Thr Val      396

CCC AGG TTC CTT ATG TGC AAC CTG GCC TTT GCT GAT CTC TGC ATT GGA     1310
Pro Arg Phe Leu Met Cys Asn Leu Ala Phe Ala Asp Leu Cys Ile Gly      412

ATC TAC CTG CTG CTC ATT GCA TCA GTT GAT ATC CAT ACC AAG AGC CAA     1358
Ile Tyr Leu Leu Leu Ile Ala Ser Val Asp Ile His Thr Lys Ser Gln      428

TAT CAC AAC TAT GCC ATT GAC TGG CAA ACT GGG GCA GGC TGT GAT GCT     1406
Tyr His Asn Tyr Ala Ile Asp Trp Gln Thr Gly Ala Gly Cys Asp Ala      444

GCT GGC TTT TTC ACT GTC TTT GCC AGT GAG CTG TCA GTC TAC ACT CTG     1454
Ala Gly Phe Phe Thr Val Phe Ala Ser Glu Leu Ser Val Tyr Thr Leu      460

ACA GCT ATC ACC TTG GAA AGA TGG CAT ACC ATC ACG CAT GCC ATG CAG     1502
Thr Ala Ile Thr Leu Glu Arg Trp His Thr Ile Thr His Ala Met Gln      476

CTG GAC TGC AAG GTG CAG CTC CGC CAT GCT GCC AGT GTC ATG GTG ATG     1550
Leu Asp Cys Lys Val Gln Leu Arg His Ala Ala Ser Val Met Val Met      492

GGC TGG ATT TTT GCT TTT GCA GCT GCC CTC TTT CCC ATC TTT GGC ATC     1598
Gly Trp Ile Phe Ala Phe Ala Ala Ala Leu Phe Pro Ile Phe Gly Ile      508

AGC AGC TAC ATG AAG GTG AGC ATC TGC CTG CCC ATG GAT ATT GAC AGC     1646
Ser Ser Tyr Met Lys Val Ser Ile Cys Leu Pro Met Asp Ile Asp Ser      524

CCT TTG TCA CAG CTG TAT GTC ATG TCC CTC CTT GTG CTC AAT GTC CTG     1694
Pro Leu Ser Gln Leu Tyr Val Met Ser Leu Leu Val Leu Asn Val Leu      540

GCC TTT GTG GTC ATC TGT GGC TGC TAT ATC CAC ATC TAC CTC ACA GTG     1742
Ala Phe Val Val Ile Cys Gly Cys Tyr Ile His Ile Tyr Leu Thr Val      556

CGG AAC CCC AAC ATC GTG TCC TCC TCT AGT GAC ACC AGG ATC GCC AAG     1790
Arg Asn Pro Asn Ile Val Ser Ser Ser Ser Asp Thr Arg Ile Ala Lys      572

CGC ATG GCC ATG CTC ATC TTC ACT GAC TTC CTC TGC ATG GCA CCC ATT     1838
Arg Met Ala Met Leu Ile Phe Thr Asp Phe Leu Cys Met Ala Pro Ile      588

TCT TTC TTT GCC ATT TCT GCC TCC CTC AAG GTG CCC CTC ATC ACT GTG     1886
Ser Phe Phe Ala Ile Ser Ala Ser Leu Lys Val Pro Leu Ile Thr Val      604

TCC AAA GCA AAG ATT CTG CTG GTT CTG TTT CAC CCC ATC AAC TCC TGT     1934
Ser Lys Ala Lys Ile Leu Leu Val Leu Phe His Pro Ile Asn Ser Cys      620

GCC AAC CCC TTC CTC TAT GCC ATC TTT ACC AAA AAC TTT CGC AGA GAT     1982
Ala Asn Pro Phe Leu Tyr Ala Ile Phe Thr Lys Asn Phe Arg Arg Asp      636

TTC TTC ATT CTG CTG AGC AAG TGT GGC TGC TAT GAA ATG CAA GCC CAA     2030
Phe Phe Ile Leu Leu Ser Lys Cys Gly Cys Tyr Glu Met Gln Ala Gln      652

ATT TAT AGG ACA GAA ACT TCA TCC ACT GTC CAC AAC ACC CAT CCA AGG     2078
Ile Tyr Arg Thr Glu Thr Ser Ser Thr Val His Asn Thr His Pro Arg      668

AAT GGC CAC TGC TCT TCA GCT CCC AGA GTC ACC AAT GGT TCC ACT TAC     2126
Asn Gly His Cys Ser Ser Ala Pro Arg Val Thr Asn Gly Ser Thr Tyr      684

ATA CTT GTC CCT CTA AGT CAT TTA GCC CAA AAC TAAAACACAA TGTGAAAATG  2179
Ile Leu Val Pro Leu Ser His Leu Ala Gln Asn                        695
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FIG. 4 (2 of 2)

FIG. 5

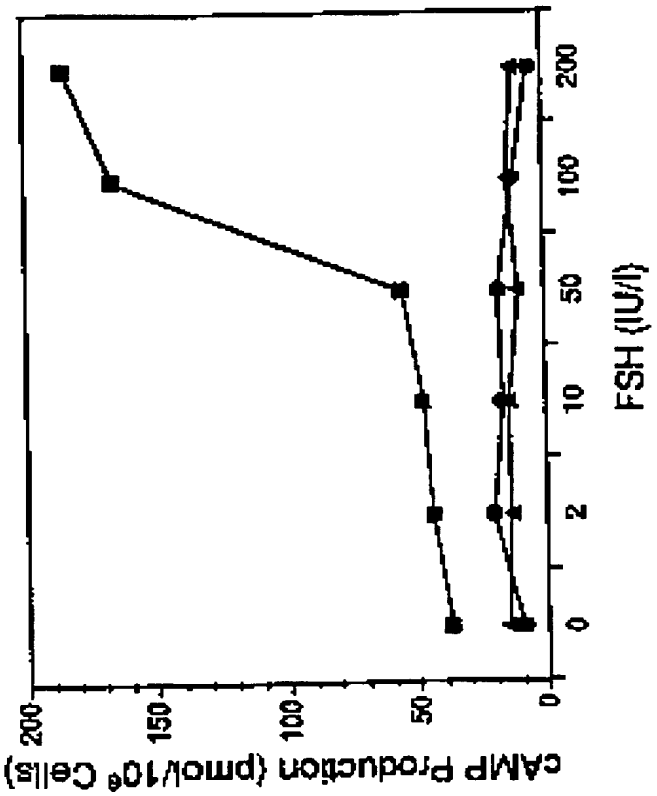

FIG. 6